BIOCHEMICAL AND MOLECULAR GENETIC STUDIES ON MITOCHONDRIAL ATPase

Ian Connerton B.Sc. (HONS), M.Sc. (Warwick University)

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Department of Chemistry and Molecular Sciences
University of Warwick

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SUMMARY

An attempt has been made to rationalise the multi-faceted inhibitory actions of trialkyltins on mitochondria, including studies on their inhibition of ATP synthase and concomitant depolarisation of mitochondrial membrane potential in rat liver mitochondria. These functions have been shown to occur in the absence of halide/hydroxyl exchange. The majority of trialkyltin compounds studied here show a concentration dependent differential inhibition of the ATP hydrolase and ATP synthase functions of the mitochondrial ATPase complex. The relative trialkyltin concentrations at which these events take place, are in the order of ATPase > ATP synthase $\geq \Delta p$ reduction.

A comparison has been made between the n-alkyltins and internally penta-coordinated alkyl tins, revealing penta-coordinacy per se does not give rise to potent inhibitory action. However, the internally nitrogen-tin coordinated species (2- (dimethylamino) methyl diethyltin halide, provides an excellent specific inhibitor of the mitochondrial ATPase complex.

The relative sensitivities to the mitochondrial ATPase directed drugs, oligomycin and ossamycin, of wild type and drug specific mutants of the yeast Saccharomyces cerevisiae has been assessed both in vivo and in vitro. DNA sequencing studies of mutant and wild type mitochondrial DNAs have established the localisation of the mutant alleles $Oli^R 2\text{-}76$ and $Oss^R 1\text{-}92$, and their respective predicted aminoacid residue changes: methionine to phenylalanine and aspartic acid to asparagine, in subunit 6 of the OS-ATPase complex. Wild type DNA sequence has also revealed the genes for subunit 8 of OS-ATPase and a putative maturase sequence, downstream of the Oli 2 gene.

ABBREVIATIONS

A	Adenosine nucleotide
ADP	Adenosine diphosphate
ANS	1-analino-8-napthalene sulphonate (magnesium salt)
Ant.A	Antimycin A
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAP	Bacterial alkaline phosphatase
BCIG (X-gal)	5-bromo-4-chloro-3-indolyl-2-D-galactoside
bp	Base pair
BSA	Bovine serum albumin
C	Cytidine nucleotide
CCCP	Carbonyl cyanide m-chloro phenyl hydrazone
CIP	Calf intestinal phosphatase
CT DNA	Calf thymus DNA
DAPI	4', 6-diamidino-2-phenyl indole dihydrochloride
DBCT	Dibutylchloromethyltin chloride
DCCD	N, N'-Dicyclohexyl-carbodiimide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNP	2, 4-dinitrophenol
dATP	deoxy Adenosine triphosphate
dCTP	deoxy Cytidine triphosphate
dGTP	deoxy Guanidine triphosphate
dNTPs	deoxy nucleotides
dTTP	deoxy Thymidine triphosphate
ddATP	dideoxy Adenosine triphosphate
ddCTP	dideoxy Cytidine triphosphate
ddGTP	dideoxy Guanidine triphosphate
ddNTP	dideoxy nucleotides
ddTTP	dideoxy Thymidine triphosphate
dsDNA	Double stranded DNA
$DSEP^+$	dimethylstyryl 1-ethyl pyridinium cation
$DSMP^+$	dimethylstyryl 1-methyl pyridinium cation
DTT	Dithiolthreitol
EDTA	Ethylene diamine-tetra-acetic acid
EtBr	Ethidium bromide
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenyl
G	Guanidine nucleotide
IPTG	Isopropyl-2-D-thiogalactopyronoside
Kbp	Kilo base pair
LMP agarose	Low melting point agarose
mtDNA	Mitochondrial DNA
mt-genome	Mitochondrial genome
mt-RNA	Mitochondrial RNA
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
OSCP	Oligomycin sensitive conferring protein
O.S.ATPase	Oligomycin sensitive ATPase
OLI (Oli)	Oligomycin
ORF	Open reading frame
Oss	Ossamycin
O.D.	Optical density

PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Pi	Inorganic phosphate
p.s.i.	Pound per square inch
Quin.Red	2-([2-(4-dimethylamino)-phenyl]ethyl)-1-ethyl Quinaldine
RF	Replicative form
Rh6G	Rhodamine 6G
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rDNA	Ribosomal DNA
r.p.m.	Revolution per minute
RNase	Ribonuclease
ρ	Rho factor, loss of which involves deletion in mt-DNA leading to the 'petite' phenotype (ρ^- or ρ^0)
S	Sedverg's constant
SDS	Sodium dodecyl sulphate
SMP	Sub mitochondrial particles
ssDNA	Single stranded DNA
SSC	Standard saline citrate
Succ	Succinate
1799	1, 1-bis (hexafluoro acetonyl) acetone
T	Thymidine nucleotide
TBE	Tris-borate EDTA buffer
TBT	Tributyltin
TCyT	Tricyclohexyltin
TEMED	N, N, N', N', tetramehtyl ethylene diamine
TET	Triethyltin
TMT	Trimethyltin
TPhT	Triphenyltin
$TPMP^+$	Methyltriphenylphosphonium cation
TPrT	Tripropyltin
URF	Unknown reading frame
UV	Ultra violet
Val	Valinomycin
VEN	Venturicidin
Ve2283	(2-[(dimethylamino) methyl] diethyltin halide

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Studies on the mechanisms of energy conservation in living cells has proved a major challenge to biochemists. The central ideas of cellular electron transport liberating energy, which is in turn utilised for the production of ATP as functional biological units of energy are now firmly established. These processes appear to remain fundamentally similar, whether they are associated with the inner mitochondrial membranes of eukaryotic cells, the thylakoid membranes of chloroplasts in plant life, or plasma membranes of prokaryotic cells.

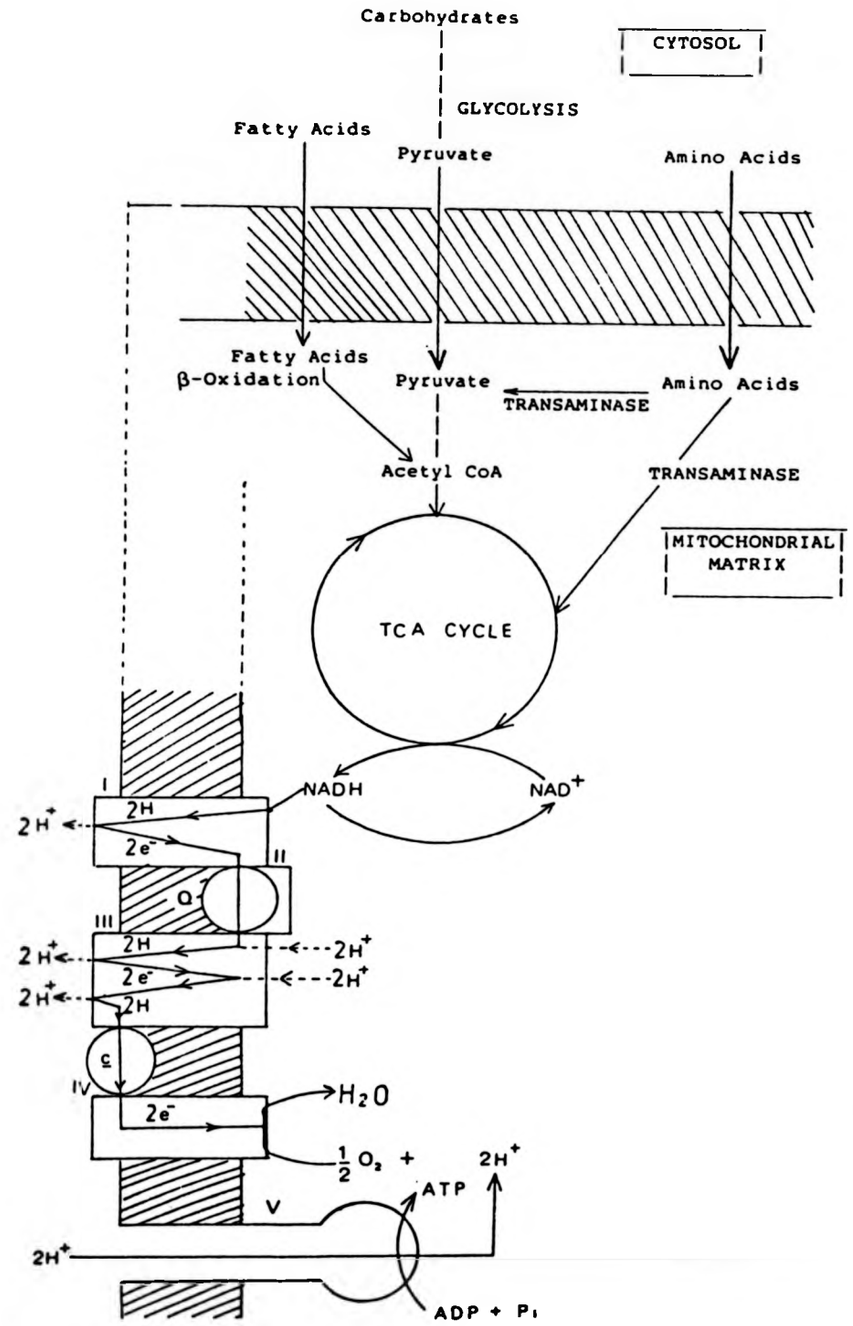
The present thesis is concerned with the ATPase complex which has the capacity to synthesise ATP using the energy derived from the oxidation of reducing equivalents by the respiratory chain, or hydrolyse ATP to release free inorganic phosphate and the equivalent energy from breaking of the phosphate anhydride bond. Before discussing these functions further, the structure and function of the respiratory chain will be outlined briefly (see Chance et al, 1969a and 1969b for in-depth reviews).

1.2 The Respiratory Chain

The components of the respiratory chain and their order are represented schematically in Figure 1.i. This information is a result of several lines of evidence, based on the measurements of redox potential of various individual components (Chance et al, 1969a), kinetic determination of the reaction sequence (Chance et al, 1969a and b), studies on the donor and acceptor specificity of isolated components (Chance et al, 1969a) and the interaction of electron transfer inhibitors with the electron transport chain (Onishi, 1973).

The protein fractionation studies of Hatefi et al, 1962a, b and c, have shown that the respiratory chain can be split into four complexes

Figure 1.i. The relationship among the major oxidative pathways of mitochondria and their ultimate connection to the terminal ATP generating proton pump (the ATP synthase or the OS-ATPase or the Complex-V). I, II, III, IV, and V are various respiratory complexes found in the mitochondrial inner membrane (see Hatefi et al, 1962; Racker, 1979). I, NADH - UQ oxidoreductase; II, Succinate dehydrogenase (succinate UQ reductase); III, UQH_2 - Cytochrome c reductase (cytochrome bc_1 complex; IV, Cytochrome c oxidase; V, ATP synthase or the OS-ATPase.



designated complex I, II, III, and IV catalysing the following partial reactions:-

I - NADH - ubiquinone reductase
$NADH + Q + H^+ \rightarrow NAD^+ + QH_2$

II - Succinate ubiquinone reductase
$Succinate + Q \rightarrow Fumarate + QH_2$

III - Ubiquinol cytochrome C reductase
$QH_2 + 2\ ferricyt.\ c \rightarrow Q + 2\ ferrocyt.\ c$

IV - Cytochrome c oxidase
$2\ ferrocyt.\ c + 2H^+ + \frac{1}{2}O_2 \rightarrow 2\ ferricyt.\ c + H_2O$

These complexes may also be reconstituted stoichiometrically to produce a reconstituted electron transfer chain which behaves similarly to that found in intact mitochondria with respect to respiratory inhibitors. Redox potential measurements on both in situ and isolated respiratory complexes have enabled the electron carriers to be positioned on a potential diagram (Chance, 1972 and 1977). The respiratory components have been associated with three levels of mid-potentials where the carriers are clustered, between these groups are gaps or spans where electron movements equate to large decreases in free energy, each sufficient to provide the necessary energy for the phosphorylation of ADP with inorganic phosphate. These changes in free energy have been designated as sites I, II and III, which represent the spans between NADH and co-enzyme Q; cyt.b and cyt.c; cyt.a and oxygen respectively (shown diagramatically in Figure 1.ii). The electron carriers with fixed mid-potentials (or isopotential pools) have been suggested to be linked by carriers of variable potential at sites I, II and III. These have been proposed to be Fe-S)N_2, cyt.b_T and cyt.a_3 respectively, based on the findings that their mid-potentials were ATP and pH dependant (Chance, 1972 and 1977). The isopotential pools act as redox ballasts which allow rapid equilibrium of electron transfer reactions which are independent of energy coupling reactions. Therefore,

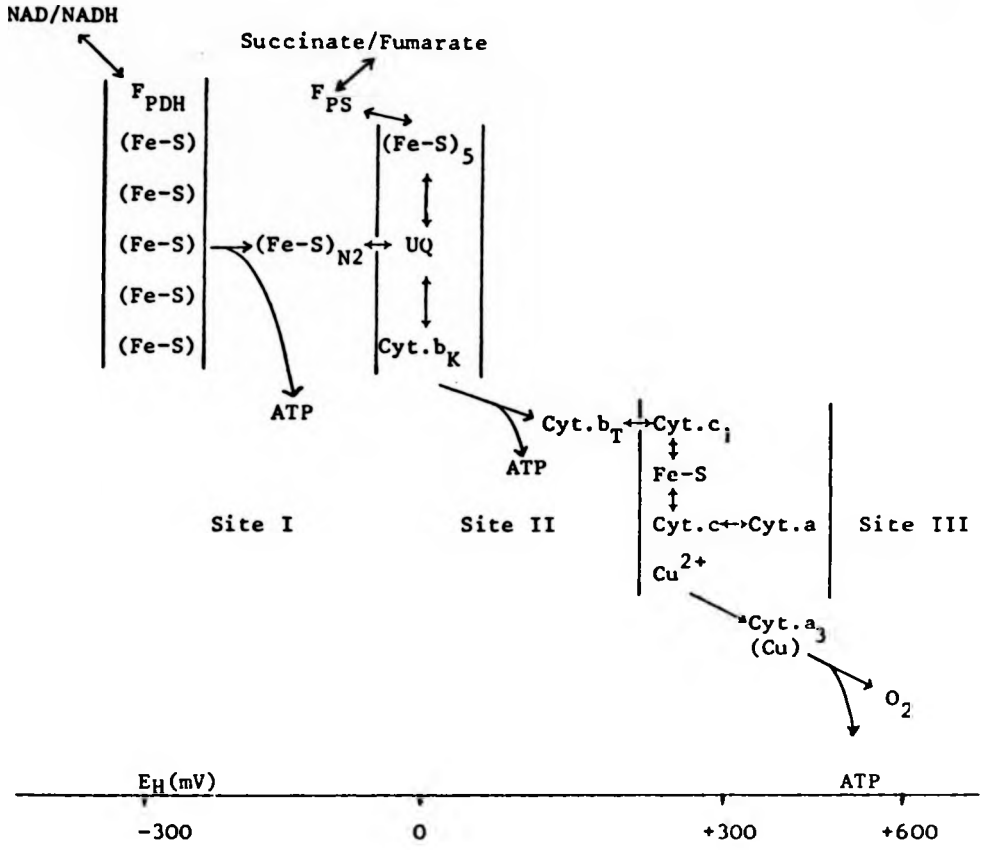


Figure 1.ii

The electron carriers of the respiratory chain arranged as a group of fixed potential (or isopotential 'pools') and individual components of variable mid-potentials (Modified from Chance, 1972).

energy transduction via the respiratory chain occurs through a series of quasi-equilibrium steps. For example, cyt.b_T may reside as four species, oxidised high mid-potential, oxidised low mid-potential, reduced high mid-potential and reduced low mid-potential. As the low mid-potential form cyt.b may only react with group II carriers and in its high mid-potential form can only react with group III carriers. The sites of energy conservation appear physically unrelated, therefore, consideration must be given as to how the fall in potential via the respiratory chain and subsequent release of energy is related to the synthesis of ATP via complex V or the ATP complex.

1.3 Oxidative Phosphorylation

Oxidative phosphorylation is the process whereby the energy released from the electron transport chain is made available to the ATP complex. This relationship has been described under three broad headings:

1. Chemical Intermediate Theory (Slater, 1953)
2. Chemiosmotic Hypothesis (Mitchell, 1961)
3. Conformational Theory (Boyer, 1965)

The central common factor to each of the above theories is the function of a high energy intermediate from the respiratory chain which may drive ATP synthesis, transhydrogenation, ion transport as well as ATP driven reversed electron transport via the ATPase complex. However, the above theories differ fundamentally in the nature of the high energy intermediates involved.

1. The Chemical Intermediate Theory

The chemical theory as originally proposed by Slater, 1953 was based on a mechanism of substrate level phosphorylation (Warburg and Christian, 1939). The basis of the theory is the formation of high energy chemical intermediates by the redox reaction of the electron transport chain, which may in turn, provide the energy to drive ATP synthesis or other

energy-linked reactions. Chance and Williams, 1956 have postulated the following reaction series involved in energy transduction via chemical intermediates.

1. $AH_2 + B + I \rightleftharpoons A \sim I + BH_2$
2. $A \sim I + X \rightleftharpoons A + X \sim I$
3. $X \sim I + Pi \rightleftharpoons X \sim P + I$
4. $X \sim P + ADP \rightleftharpoons X + ATP$

AH_2 and B are two adjacent respiratory carriers and '$\sim$' refers to a high energy bond responsible for energy transmission (possibly an anhydride or thioester bond). The natures of X and I are unknown. X~I is regarded as the common energy transducing intermediate and the possible target of uncoupling agents which, directly or indirectly, catalyse its hydrolysis (Slater, 1966). Evidence for the chemical hypothesis is largely based on the actions of various inhibitors, for example oligomycin which is thought to block reaction 3 in the above scheme, possibly sited at the ATPase complex (Lardy et al, 1958; Lardy and McMurray, 1958 and Slater, 1966). Further evidence for the chemical hypothesis is derived from the observations that partial reactions such as transhydrogenation and reversed electron transfer can be driven either by respiration or ATP. However, the chemical hypothesis makes no provision for the observations that the integrity of the mitochondrial membrane appears to be a prerequisite for ATP synthesis, and the fact that a wide range of uncouplers with very different chemical structures are quite active. The theory also suffers from the failure of various workers to identify the proposed ubiquitous intermediate molecules (Greville, 1969 and Harold, 1972).

2. The Chemiosmotic Hypothesis

The chemiosmotic hypothesis was proposed by Mitchell in 1961, suggesting the intermediate in energy transduction was a proton gradient across the membrane. The chemiosmotic hypothesis is based on the following

four main postulates and is further summarised in Figure 1.iii

1. The ATP synthase is a chemiosmotic membrane located reversible proton translocating ATPase, having a characteristic H^+/P stoichiometry.
2. The respiratory chain is a membrane located vectorial metabolic proton translocating system, having a characteristic $H^+/2e$ stoichiometry and having the same polarity of proton translocation across the membrane under forward redox activity as the ATPase during the hydrolysis of ATP.
3. There are proton (or hydroxyl) linked solute porter systems for osmotic stabilisation and metabolite transport.
4. Systems 1-3 are vectorially arranged through a topologically closed insulating membrane, called the coupling membrane, which is impermeable to ions, particularly to H^+ and OH^-.

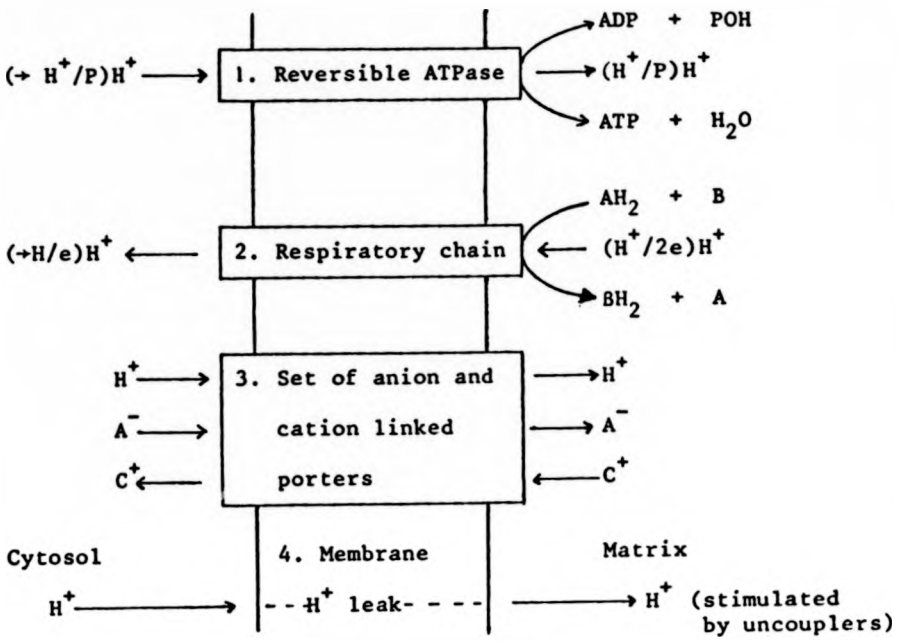
For further review see Mitchell, 1979.

The central dogma of th chemiosmotic hypothesis is based upon the generation of a proton electro-chemical potential (μH^+) by the electron transfer chains of mitochondria, chloroplasts or bacteria, which in turn drives the synthesis of ATP via the passage of protons to produce a protonic circuit. The driving force of ATP synthesis has been defined as the proton motive force (Δp) which is the sum of the pH gradient (ΔpH) and the membrane potential ($\Delta \Psi$), which are facilitated by the proton pumps of the electron transfer chains of respiration or photosynthesis, in accordance with the following equation (Mitchell, 1961; 1966; 1968; 1976; 1977a; 1977b; and 1979):

$$\frac{\mu H^+}{F} = \Delta p = \Delta \Psi - 2.3 \frac{RT}{F} \Delta pH$$

As the protonic circuit is completed by the passage of protons through the ATPase complex there should be a distinct stoichiometric coupling

Figure 1.iii



The Fundamental Postulates of the Chemiosmotic Theory

(Mitchell, 1966)

between the oxidation/reduction of the electron transport chain and the phosphorylation. The P/O (P/2e) quotient of the overall process is given by the $H^+/2e$ quotient of the redox system divided by the H^+/P quotient of the ATPase, i.e. $P/2e = (H^+/2e)/(H^+/P)$. A short circuit of the proton pathway or effective leakiness of the proton impermeable membrane would collapse Δp, uncoupling phosphorylation and allowing the uncontrolled acceleration of redox activity. Such a phenomenon is thought to occur on the addition of an uncoupler to the system. Uncouplers are thought to effect their action by allowing the free diffusion of protons across the lipid bilayer either as the lipid soluble protenated acid or the conjugate base of the compound (eg. 2, 4- dinitrophenol or DNP). The existence of uncouplers had been described before the chemiosmotic hypothesis had been formulated and the demonstration that a number of these compounds increased protonic conductance through synthetic bilayers was considered an important piece of evidence in favour of the theory (for reviews of the activities and functions of uncouplers and ionophores, see Hendersen, 1971 and Ovchinnikov, 1979).

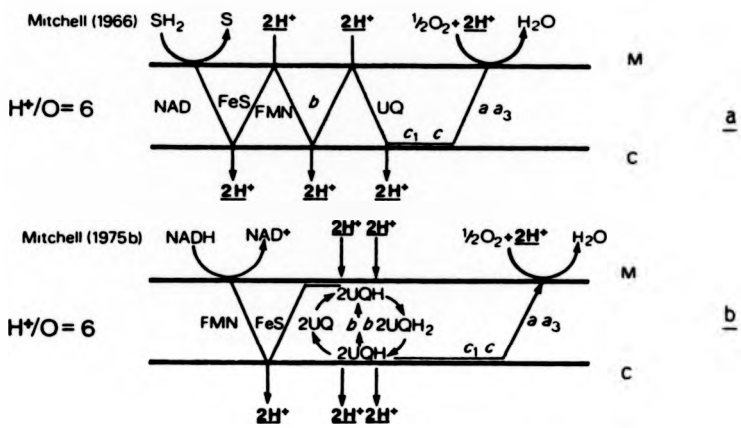
The chemiosmotic hypothesis has gained wide ranging acceptance over the last 20 years, however, at its conception it stimulated much debate between its advocates and those of the chemical theory (see Boyer et al, 1977). The result of which has been a great deal of research aimed at validation, or otherwise, which has led to the accumulation of a lot of data. Such data which may be ascribed to evidence derived from studies on mitochondria, submitochondrial particles and reconstituted vesicles is summarised below.

1. Vectorial, transmembrane movement of H^+ and other ions accompany both respiration and ATP hydrolysis (De Pierre and Ernster, 1977; Garland, 1977; Hauska and Trobt, 1977; Croft and Wood, 1978; Rottenberg, 1975).

2. Most of the energy transducing systems can generate a proton gradient and/or a membrane potential across the membrane in which they are located (Rottenburg, 1975).
3. Energy transfer may take place within membrane components *via* a proton gradient and/or membrane potential (Rottenburg, 1975).
4. Uncouplers may increase proton flux in membranes (Henderson, 1971; Haydon and Hladky, 1972 and McLoughlin, 1972).
5. Artificially generated electrochemical gradients are kinetically competent in driving ATP synthesis (Jaggendorf and Uribe, 1966; Thayer and Hinckle, 1975 and Rottenburg, 1979).

Further, Mitchell has suggested that the intermediate proton gradient is generated by the action of H^+ translocating 'redox loops', formed by alternating hydrogen carriers and electron carriers of the respiratory chain (see Figure 1.iva) (Mitchell, 1966). However, such a mechanism has attracted serious criticism, Harman *et al*, 1974 have pointed out that although complexes I and III contain both hydrogen (NAD, FMN, CoQ) and electron carriers (Fe-S, cyt.b and cyt.c), (unlike complex IV, which has only electron carriers), they do not form transmembrane loops. This evidence led Mitchell to propose the 'protonmotive ubiquinone cycle' (or 'Q cycle') for proton translocation (see Figure 1.ib) (Mitchell, 1975 and 1976). However, the 'Q cycle' requires the free diffusion of Q and QH_2 through the membrane, and a certain distribution of b cytochromes, which has been questioned (De Pierre and Ernster, 1977). The mechanisms by which the respiratory chain and the ATPase complex transport protons and the subsequent H^+/site quotient have generated considerable debate. Our current understanding is that the translocation of protons proceeds indirectly *via* a 'proton pump' linked to the turnover of the enzyme, evidence derived from studies on membrane topology of the catalysts involved and the measurement of $H^+/2e^-$ and H^+ /ATP

Figure 1.iv



Hypotheses for Proton Translocation by Electron-Transfer Chains

(Adapted from Nicholls, 1982).

a. Alternating hydrogen and electron carriers of the respiratory chain (Mitchell, 1966).

b. The proton motive ubiquinone cycle (or 'Q cycle') for proton translocation by electron carriers (Mitchell, 1975).

ratios (for reviews see Ernster, 1977; Reynafarje et al, 1979 and Wikstrom and Krab, 1980). A basic requirement for a model redox proton pump is that it should be compatible with the observed H^+ ejection stoichiometries, the loop hypothesis requires a H^+/site ratio of 2. Results supporting these predictions have been reported by Mitchell and Moyle (Mitchell and Moyle, 1965; 1967; 1968 and Moyle and Mitchell, 1973), who have shown that electron transport from NADH to oxygen results in a translocation of $6H^+$. However, other researchers have observed significantly higher H^+/site ratios, often exceeding 3, for both the electron transport and ATPase systems (see reviews: Brand, 1977; Reynafarje et al, 1979 and Wikstrom and Krab, 1980). These results are not consistent with 'redox loops' but may be accommodated into a model of a more general redox proton pump (see Papa, 1976 and Ernster, 1975).

As the chemiosmotic hypothesis emerged in 1961, a second theory of energy coupling was also proposed by Williams, which envisaged the proton and charge separations of chemiosmosis but remained fundamentally distinct by virtue that the protons remain associated with the membrane and do not interact with the bulk aqueous phase (see reviews by Williams, 1976 and 1978a). The authors have been unable to agree on this difference, the debate on delocalised versus localised proton pathways is presently rife (see Mitchell, 1977, 1985 and Williams, 1978b, 1985).

In Williams' original hypothesis the energy of the redox reactions could be transferred to the ATPase complex as an anhydrous (or partially hydrated) proton which moves in the membrane through a channel of fixed water molecules, which is connected to the ATPase. Thus, during of ATP from ADP and Pi, the H^+ moves so as to pull H_2O molecules from the polyphosphate condensation site into the diffusion channel. The above hypothesis has certain distinct advantages over that of the

chemiosmotic counterpart, namely a more efficient utilisation of energy, better central circuits, and the possibility of discriminative coupling to various energy-linked processes. However, authors have described how delocalised protons and imposed diffusion potentials may drive ATP synthesis (Paden and Rottenberg, 1973; Azzone et al, 1978 and Thayer and Hinckle, 1978), and as Mitchell has pointed out, the hypothesis is limiting in the respect that it is difficult to experimentally falsify. More recently, Kell (1979) has proposed his 'electrodic hypothesis' where a protonic circuit is set up between aqueous/membrane phases on either side of the bilayer completed by a proton translocating ATPase. This scheme envisages only limited interaction between the translocated protons and the bulk aqueous phase.

The evidence for and against a truly delocalised chemiosmotic mechanism or a localised proton pathway in energy coupling has recently been reviewed at length by Ferguson, 1985.

3. Conformational Theory

The conformational theory as originally proposed by Boyer, (1965), proposed that the energy generated by electron transport was conserved as a 'high energy' conformational state of the respiratory carrier. This energy was in turn utilised to drive ATP synthesis.

$$AH + B \rightleftarrows A^* + BH_2$$
$$A^* + ADP + Pi \rightleftarrows A + ATP + H_2O$$

Support for the theory was based on the observation that mitochondrial structure was energy dependent (Green, 1970; and Hackenbrock, 1966) and electron flow in bacteria, chloroplasts and mitochondria is accompanied by changes in the conformation of their F_1 ATPase (Boyer, 1977; Ryrie and Jagendorf, 1972; McCarty and Ferguson, 1973; and Bertina et al, 1973). However, little evidence has arisen to support a direct interaction between the redox centres and the ATP complex.

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Later, a variation on this hypothesis was proposed, that ATP synthesis occurred at the active site of the ATPase prior to the input energy, which necessitated the release of the newly formed ATP molecule from the ATP synthase via an energy dependent conformational change (Boyer, 1977; and Harris and Slater, 1975). This hypothesis was based on the findings that the exchangeability of adenine nucleotides bound to F_1 was dependent on the flow of electrons (Harris and Slater, 1975) and on the insensitivity of the phosphate anhydride formation to uncouplers of oxidative phosphorylation (Boyer et al, 1975). However, the mechanisms of the exchange reactions cannot easily be reconciled with Mitchell's proposals that protons are directly involved in the anhydride bond formation (Mitchell, 1977).

More recently, evidence for a conformational change during ATP binding has come from studies with isolated F_1 ATPase modified with inhibitors such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), dicyclohexyl carbodimide (DCCD) and dialdehyde ATP derivatives (Kohlbrenner and Boyer, 1982; Rojendra et al, 1983 and Kumer et al, 1979) modified F_1 ATPase has been shown to be functionally catalytic in ATP synthesis but only experiencing slow reactivation of hydrolase activity when reconstituted on submitochondrial particles stripped of F_1 ATPase (Soong and Wang, 1984; and Matsuno-Yagi and Hatefi, 1984). Experiments such as these, and studies on the binding of tritiated nucleotides to modified enzymes has also led to arguments about the nature of the β-subunit of F_1 ATPase, whether they act independently or cooperatively (Grubmeyer et al, 1982; Wang, 1985; and Rajendra et al, 1985).

1.4 The Mitochondrial ATPase Complex

The mitochondrial ATPase complex has been resolved into three basic segments, based on either their enzymatic function, sensitivity to various inhibitors or the physical properties of the constituitive polypeptide(s) (shown schematically in Figure 1.v).

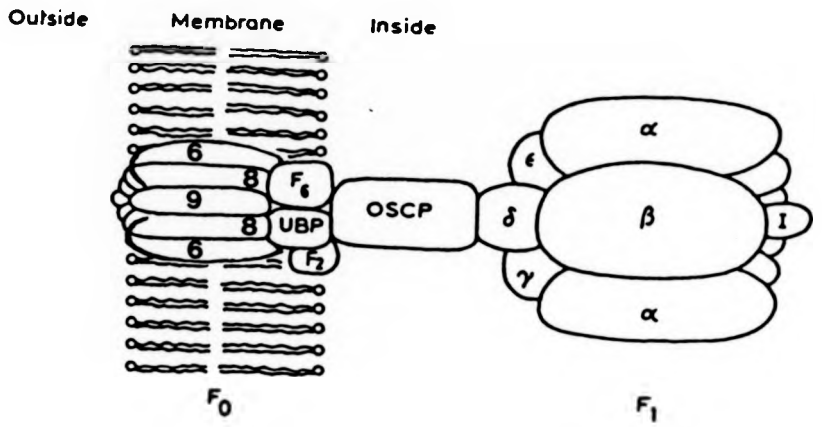


Figure 1.v The possible structure of the O.S.ATPase or ATP synthase. The scheme is hypothetical. α, β, γ, δ, and ϵ are various subunit components of the F_1 segment. 6, 8, 9 are three recognised subunits of the F_0 sector. The Oligomycin-sensitivity conferring protein (OSCP), the uncoupler binding protein (UBF), Factor-6 (F_6), Factor-B (F_2) have been tentatively placed in the diagram. I is the ATPase inhibitor protein which probably remains attached to the β subunit.

1. Factor 1 (F_1):- an oligomycin insensitive cold labile and water soluble ATPase; sensitive to aurovertin and NBD (C1)

2. Factor O (Fo):- The water insoluble membrane sector of the enzyme which remains buried in the lipid bilayer and may provide an ion channel for the re-entry of protons as envisaged by the chemiosmotic hypothesis, probably the binding sites for DCCD, oligomycin, venturicidin and trialkyltins which effect the activities of the halo-enzyme or oligomycin sensitive ATPase (OS-ATPase).

3. O.S.C.P. (Oligomycin-sensitivity conferring protein):- a water soluble protein that serves to provide the link between the F_1 and Fo segments.

Additionally, a small peptide inhibitor of ATP hydrolytic activity has been characterised (Pullman and Monroy, 1963; Ebner, 1974). Properties of the various coupling factors associated with the ATPase complex are summarised in Table 1.i.

a) F_1 ATPase

F_1 ATPase consists of five subunits: α, β, γ, δ, and ϵ (see Table 1.ii) with the general stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ (see Crass, 1981). The complex may be isolated by mechanical disruption, or by chloroform extraction. Isolated F_1 has only ATP hydrolase activity and remains oligomycin insensitive. The β subunit has been proposed to contain the catalytic site, based on reconstitution and inhibitor binding experiments (Yoshida, et al, 1977; Ferguson, et al, 1974 and 1975; Ho and Wang, 1983; Soong and Wang, 1985). Biogenesis experiments have confirmed that all five of these subunits are synthesised on cytoribosomes and imported into the mitochondria (Tzagoloff, et al, 1973; Tzagoloff and Meagher, 1971).

b) O.S.C.P.

The oligomycin sensitivity conferring protein is a small basic protein of approximately 20 K Daltons, belonging to the mitochondrial ATPase complex (Tzagoloff, et al, 1968; MacLennon and Tzagoloff, 1968; Senior,

Table 1.1. Properties of Coupling Factors of Oxidative Phosphorylation

Factor	Alternate name	Mol.wt	Intrinsic activity	Functions *in vitro*
F_1		360,000	ATPase	Hydrolysis of ATP, Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
F_0		200,000	None	Proton translocation
F_6	F_c	8,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
OSCP		18,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
Factor B	F_2	12,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation. Stimulation of ATP-P_i exchange in O.S.ATPase

Legend Table 1.ii

a. Tzagoloff et al, 1973.
b. Serrano et al, 1976.
c. De Jong et al, 1979.
d. Devlin, 1982.
e. Koch, 1976.

Table 1.ii Components of the OS-ATPase Complex

Subunit	Molecular Weights					Component
	Yeast a	Beef Heart b	Rat Liver c	Sea Urchin d	X. laevis e	
α	58,000	60,000	53,000	55,000	56,000	F_1
β	55,000	56,000	49,000	52,000	53,000	F_1
	33,000				32,000	
γ	29,000	34,000	34,000	39,000	41,000	F_1
δ	14,000	14,000	12,000	17,000	17,500	F_1
ε	8,000	11,000	9,000	10,000	11,500	F_1
5	28,000	32,000	32,000	31,000	32,000	F_o
6	22,000	23,000	25,000	23,000	24,000	F_o
7	18,000	20,000	22,000	17,000		OSCP
8	12,000					F_o
9	7,500	8,000	7,500			F_o

1971; Senior, 1979; and Glaser et al, 1980). The mediated interaction between the F_1 and Fo segments of the ATPase complex has been probed by the ability of oligomycin, a ligand of the Fo fraction, to inhibit ATPase activity of F_1 (Van De Stadt et al, 1972; and Vadineanu et al, 1976).

c) Fo Section

The Fo components of the OS-ATPase complex is the least understood part of the enzyme complex. Even the number of protein subunits is still under debate, let alone the stoichiometries of the components, all this controversy about an apparently abundant protein constituting 2% of the organelle mass (Pederson et al, 1978). Early biogenesis studies with yeast using radio-labelled aminoacids in the presence of cycloheximide, so as to prevent cytoribosome protein synthesis, allowed four mitochondrially synthesised polypeptides to be immuno-precipitated with the holoenzyme using antisera raised against F_1 (Tzagoloff and Meagher, 1972). These were identified as OS-ATPase subunits of approximate molecular masses 29, 22, 12 and 7.5 K Daltons (see Table 1.ii). Of these original proposed mitochondrially encoded Fo components, the 22, 12 and 7.5 K Dalton proteins have been confirmed and further identified at the mtDNA level as bona fide mitochondrial genes (Hensgens et al, 1979; Macino and Tzagoloff, 1979 and 1980; Novitski et al, 1984; and Ray, 1985, see also Chapter 7 of this thesis), namely, subunits 6, 8 and 9 (or the DCCD binding protein) respectively. Subunits 8 and 9 have also been corroborated by full length aminoacid sequences (Sebald et al, 1979; and Velours et al, 1984). The ubiquity of the Fo components has also been established as homologous predicted aminoacid sequences have been identified in other fungi, higher plants, insects and mammals (for review see Ray, 1985). The 29 K Dalton protein equivalent in beef heart has been proposed to be necessary for oligomycin

sensitive ATP- ^{32}Pi exchange and possesses sensitive titratable SH groups (Zimmer et al, 1979 and 1982; and Baumert et al, 1981), however whether the 29 K Dalton protein(s) is essential for high ATP- ^{32}Pi exchange and/or the structural integrity of the OS-ATPase complex has been questioned (Joshi and Tarok, 1984).

Two other recognised coupling factors exist which may be membrane-bound: F_6 (Kanner et al, 1976), and Factor B (see review by Sonadi, 1982). F_1 has been purified and shown to be involved in the binding od F_1 to Fo as well as in the restoration of oxidative phosphorylation and ATP dependent reactions in depleted particles (Kanner et al, 1976). Recently, the primary aminoacid sequence of F_6 has been reported (Fang et al, 1984). F_6 is probably a membrane protein with charged residues clustered in the aqueous phase separated by hydrophobic segments of 9.006 K Daltons molecular weight. Factor B has also been purified (Apparent molecular mass 15 (Sonadi, 1982) or 11 K Daltons (You and Hatefi, 1976), which contains essential vicinal dithiols (Stigall et al, 1979; and Joshi and Hughes, 1981) which may prevent its function in oxidative phosphorylation if treated with sulphydryl reagents; as a consequence of this, Factor B will bind the ligand Cd^{2+} (Kaplay et al, 1984; Sonadi et al, 1984; Lakshmikantham et al, 1984; and Yagi and Hatefi, 1984). It has been suggested that the protein may exert its effect by decreasing the permeability of the membrane to protonic movements.

1.5 Genetics of Oxidative Phosphorylation

The genetics of oxidative phosphorylation has been focused on two organisms which may sustain the serious physiological disadvantage of being unable to grow on non-fermentable media, Escherichia coli representing prokaryotes, and the yeast Saccharomyces cerevisiae for eukaryotes. The E. coli enzyme is a simpler unit, consisting of the

five F_1 subunits (analogous to its mitochondrial counterparts discussed in the previous section, however the δ subunit has shown some homology with the eukaryotic OSCP) and three Fo subunits, namely a, b and c. Mutants of these genes have isolated and complemented, all the genes appear linked and subsequent DNA sequence analysis has revealed an atp operon consisting of all eight genes and an extra reading frame, I, of unknown function (see reviews by Hoppe and Sebald, 1984 for protein studies, and Walker et al, 1984, for DNA sequence analysis).

Mutants affecting oxidative phosphorylation in S. cerevisiae fall into two phenotypes, drug resistant mutants of ATPase inhibitors and uncouplers (see Table 1.iii from Griffiths, 1976) and those strains which are unable to grow on oxidative media (see Tzagoloff, 1982). Both sets of mutants may be nuclear or mitochondrially inherited, the latter group being discriminated genotypically as mit^-, a small lesion in a specific mitochondrial gene, and pet^-, a nuclear mutant allele unable to grow on oxidative media. However, it must be noted that these phenotypes are also common to those affecting other primary mitochondrial functions and were primarily distinguished by enzymatic analyses of mutant mitochondrial functions (Tzagoloff et al, 1975a and b).

Mitochondrially inherited oligomycin resistant mutants were first reported by this laboratory some fifteen years ago (Avner and Griffiths, 1970). Oligomycin resistant mutants may be considered in two main classes; those which are truly mitochonrially determined (Class II) and those which display a wide range of cross resistances and are probably nuclear controlled, although undergoing apparent mitotic segregation (Class I), (Avner and Griffiths, 1973a and Lancashire and Griffiths, 1975a). Mitochondrial oligomycin resistant mutants were originally located at two unlinked loci by allelism tests (Avner

Table 1.iii Mitochondrial drugs used in the genetic studies of oxidative phosphorylation and their possible location of action (after Griffiths, 1976)

Drug	Biochemical locus of action	Resistant mutants isolated and locus
Antimycin A	Electron transport	Yes; nuclear
CCCP	Uncoupling agent	Yes; cytoplasmic
TTFB	Uncoupling agent	Yes; cytoplasmic
1799	Uncoupling agent	Yes; cytoplasmic
Oligomycin	O.S.ATPase	Yes; nuclear & cytoplasmic
Ossamycin	O.S.ATPase	Yes; cytoplasmic
Venturicidin	O.S.ATPase	Yes; nuclear, mitochondrial & cytoplasmic
Trialkyltin	O.S.ATPase	Yes; nuclear & cytoplasmic
Aurovertin	F_1 ATPase	Yes; nuclear
Dio 9	F_1 ATPase	Yes, nuclear
Rhodamine 6G	ADP translocase(?)	Yes; cytoplasmic
Bongkrekic acid	ADP translocase	Yes; nuclear & cytoplasmic
Valinomycin	K^+ transport	Yes; mitochondrial

CCCP, Carbonylcyanide m-chlorophenylhydrazone; TTFB, 4, 5, 6, 7-tetrachloro-2-trifluoromethylbenzimidazole; 1799, α, α - bis (hexafluoroacetonyl) acetone

and Griffiths, 1973b) and multifactorial crosses (Avner et al, 1973). namely, Oli 1 and Oli 2. Further genetic analysis and the use of mitochondrially inherited venturicidin resistant mutants (Ven 1) allowed the characterisation of a third genetic locus associated with oligomycin resistance, Oli 3 (Griffiths et al, 1975; and Lancashire and Griffiths, 1975b). The Oli 3 locus is closely linked to Oli 1 (typically 1% recombination) and Ven 1 suggesting they may lie on the same gene. A naturally resistant laboratory strain revealed a fourth locus, Oli 4, which was closely linked to Oli 2 (Clavilier, 1976). Isolation and characterisation of ossamycin resistant mutants (Oss 1 and Oss 2) has brought to light a fifth and final, up to press, locus for oligomycin resistance, Oli 5 (Lancashire and Mattoon, 1979). Oli 5 mutants are a rare class of oligomycin resistant mutants which are closely linked to Oli 1, Oli 3 and Ven 1. In addition to the ant^R mitochondrial mutants described above, there are two basic types of mit^- mutants, pho 1 and pho 2. Both pho 1 and pho 2 are deficient in OS-ATPase activity and are closely linked to the antibiotic resistance loci, Oli 1, Oli 3, Oli 5, Oss 2 and Ven 1 for pho 2 and Oli 2, Oli 4 and Oss 1 for pho 1 (Foury and Tzagoloff, 1976 and Coruzzi et al, 1978). All the above mutants appear to be clustered in two segments of the genome, suggesting two sites of synthesis for the components of the OS-ATPase.

More recently, DNA sequencing strategies have been employed to examine the Oli 1 and Oli 2 regions. The Oli 1 region revealed a reading frame of some 231 nucleotides (Hensgens et al, 1979; Macino and Tzagoloff 1979; and Guat Ooi et al, 1985), specifying a protein which has an almost colinear aminoacid sequence with that of isolated subunit 9 (Sebald et al, 1979) and its DCCD binding counterpart (Sebald and Wachter, 1978). The Oli 2 region reveals a reading frame specifying

a protein of 259 aminoacids and 28.25 K Daltons molecular weight (Macino and Tzagoloff, 1980; Novitski *et al*, 1984; and Ray *et al*, 1984 (see also Chapter 7 of this thesis). The mature protein appears to be a 20 K Dalton product equating to subunit 6, which appears as a highly cycloheximide resistant band on gels of the immunoprecipitated complex (Somlo *et al*, 1982 and 1985). In addition to the antibiotic resistant defined loci is a third mitochondrially encoded product: subunit 8. This gene has been defined by a series of *mit*$^-$ mutants and their revertants in a reading frame of 48 aminoacids upstream of the Oli 2 locus, which have been correlated with the aberrant mobility of an apparent 10 K Dalton protein on SDS-polyacrylamide gels (Macreadie *et al*, 1983). Further, this protein has been corroborated by the existence of a colinear aminoacid sequence obtained from a mitochondrially translated product, purified by reverse phase high pressure liquid chromotography (Esparza *et al*, 1981; and Velours *et al*, 1984).

The α and β genes of F_1 ATPase have been cloned recently, in both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, by complementation of *pet*$^-$ mutants with a transformed DNA library in a yeast shuttle vector capable of replication in yeast and *E. coli* (Saltzgaber-Muller *et al*, 1983; Takeda *et al*, 1985; and Boutry *et al*, 1983).

CHAPTER 2

2. Dimethylaminostyryl - 1 - Alkylpyridinium dyes as Fluorescent Monitors of Mitochondrial Membrane Potential.

2.1. Introduction

Membrane potential ($\Delta\Psi$) is a parameter in the joint determination with the pH gradient (ΔpH) of the electrochemical potential difference ($\Delta\mu H^+$) or proton motive force (Δp) which drives energy conversion in biological systems (Mitchell, 1968).

$$\mu H^+/F = \Delta p = \Delta\Psi - 2.3\ RT/F\ \Delta pH$$

Membrane potential has been defined as "The potential difference that would be measured between two identical electrodes positioned on opposite sides of a membrane", (Rottenberg, 1979). This idealised view must be considered within the context of the complex microstructure of systems. Thus, central to the chemiosmotic hypothesis is the generation of a transmembrane electrical potential and pH gradient by non-permeant ions. Techniques for measuring these parameters have been developed over the last decade, based on swift permeant radioactive ion separations, electrodic measurements of bulk phase changes and spectroscopic changes. (Details of these experiments have been reviewed by Rottenberg, 1979). The spectroscopic changes applied to the measurement of membrane potential fall into two categories, the intrinsic probes which are endogenous electronic components found embedded in the membranes of chloroplasts and photosynthetic bacteria, that react when placed in an electric field (Witt, 1977), and the extrinsic probes which are foreign permeant lipophilic dyes which produce spectroscopic changes under the influence of electrical potential (Waggoner, 1979). Spectroscopic probes of the mitochondrial membrane potential fall into the latter category. The nature and uses of these probes have been reviewed extensively (Azzi, 1975; Cohen and Saltzberg, 1978; Waggoner, 1979; Rottenberg, 1979; Bashford and Smith, 1979). In short,

although optical probes offer the advantage of fast time dependent assays, they suffer from difficult calibration and do not always approximate to the Nernst equation (Burckhardt, 1977; Zorratti and Azzone, 1980; Conover and Schneider, 1981; and Robertson and Rottenberg, 1983). The styryl dye 2-(dimethyl aminostyryl)-1-methyl pyridinium cation ($DSMP^+$) has been shown to be, in contrast, a faithful and quantitative reporter over the working range of mitochondrial membrane potential, $\Delta\Psi$ (Bereiter-Hahn, 1976; Rafael, 1980; and Mewes and Rafael, 1981). The basis of the method fluorescence enhancement as $DSMP^+$ enters the more lipophilic environment of the mitochondrial matrix.

$DSMP^+$ also has the advantage of being relatively unreactive, and no direct interaction has been observed with inhibitors or membrane preparations. This is not the case with ANS which undergoes a strong bathochromic reaction with trialkyltins (cf. Appendix 1 of this thesis), and will produce variant fluorescence readings dependent on relatively low concentrations of free inorganic phosphate, an unwelcome effect when dual enzymatic and fluormetric assays are to be performed on similarly phosphorylating mitochondria. However, at relatively high dye concentrations (4 nmoles $DSMP^+$/mg mitochondrial protein) uncoupling effects on oxidative phosphorylation have been reported (Rafael, 1980). The aim of this chapter is to estimate changes in mitochondrial $\Delta\Psi$ by $DSMP^+$ fluorescence, and to calibrate these changes by standard radioactive distribution assays. The application of this method to studies of the mode of action of various inhibitors of mitochondria is described, and extensive use of this technique is made in Chapters 3, 4 and 5 in studies of the effects of triorganotins and triorganoleads on energy conservation.

2.2. Materials and Methods

Materials

Sucrose was purchased from May and Baker (Pro-Anal grade). Triphenylmethylphosphonium bromide ($TPMP^+$) and 2-([2-(4-dimethylamino)-phenyl] ethyl)-1-ethyl quinolinium iodide (Quinaldine Red) were purchased from Aldridge Chemical Co. Carbonyl cyanide p-trifluoromethoxphenylhydrozone (FCCP) was purchased from Calibiochem. Trizma base, dinitrophenol (DNP), oligomycin, valinomycin, Rotenone, Antimycin A and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. UK. 2-(dimethylaminostyryl)-1-methylpyridinium bromide ($DSMP^+$) was obtained from Gallard-Schlessinger Inc. 2-(dimethylaminostyryl)-1-ethylpyridinium iodide ($DSEP^+$) was purchased from ICN Pharmaceutical Inc. Rubidium-sulphate was produced from the carbonate purchased from Fisons UK. Ossamycin was a gift from Bristol Myers Corporation and Venturicidin was a gift from Glaxo UK. Radiochemicals $^{86}RbCl$, [^3H]- TPMP Br, [^{14}C]-Sucrose, [^{14}C]-mannitol [^{14}C]-lactate and ^3H_2O were purchased from Amersham. [^3H]-DSMP I was a kind gift from Professor J. Rafael, Heidelberg. All other reagents were of 'Anal R' grade purchased from BDH.

Methods

Preparation of Rat Liver Mitochondria

Rat liver mitochondria were prepared from the livers of adult male Wistrar rats (200-300g) starved for 24 hours prior to sacrifice. Liver mitochondria were isolated by differential centrifugation, as as essentially described by Selwyn *et al*, 1970. Excised rat livers were finely chopped at 4° C and washed with ice cold 250mM Sucrose, 10mM Tris/HCl pH 7.5, 1mM EDTA before homogenisation with 2-3 passes in a glass homogeniser with teflon pestle. The homogenate was decanted into ice cooled 30ml polycarbonate tubes and centrifuged at 2k r.p.m.

in a Sorvall RC-2B centrifuge using the SS-34 rotor (800 x g) for.10 minutes at 4^{o} C. The supernatant was then decanted into fresh 30ml polycarbonate tubes and recentrifuged as above. The supernatant was similarly treated before centrifuging again at 12K r.p.m. (10K x g) for 15 minutes at 4^{o} C. The resulting supernatant was discarded and the mitochondrial pellet was resuspended and washed in 250mM Sucrose, 10mM Tris/HCl pH 7.5, before recentrifuging at 12K r.p.m. The mitochondria were finally suspended in 250mM Sucrose, 10mM HEPES pH 7.5, at 50mg/ml protein concentration and kept at 0^{o} C prior to use within 5 hours.

When mannitol determinations of internal space were performed, 250mM sucrose was replaced by 200mM mannitol; all other conditions remained similar.

Protein Determination

Mitochondrial protein was estimated by the biuret method of Gornall et al, 1949. Particulate protein was solubilised with 5% deoxycholate prior to determination.

Determination of Mitochondrial Volumes

Internal organelle space was determined by the subtraction of [^{14}C] - sugar permeable space from total space ascertained by $^{3}H_2O$. The method described by Chappell and Crompton, (1979), was employed. Mitochondria at 1mg/ml protein were incubated at 30^{o} C with shaking in open 4ml plastic pots containing 250mM Sucrose/ 220mM mannitol [^{14}C] -, 0.2µCi/ml, 10mM HEPES pH 7.5, 5mM succinate, 2mM $MgSO_4$ and 8µM rotenone with $^{3}H_2O$ (1µCi/ml). At 1 and 2 minutes 1ml aliquots were removed and centrifuged rapidly for 2 minutes in an Eppendorf microcentrifuge. A 0.1ml sample of the supernatant was taken, acidified with 10% $HClO_4$ and mixed with scintillant. The pellet was resuspended in 0.25ml of 10% $HClO_4$, repelleted and a 0.1ml sample was again mixed

with scintillant. The samples were counted repeatedly overnight at 5 minute intervals and the mean counts per minute determined for each channel during dual counting in a Packard Tricarb liquid scintillation counter. Internal space was given by the water permeant space minus the sugar permeant space, as below:-

$$\text{Internal Space} = VS \left(\frac{^3Hp}{^3Hs} - \frac{^{14}Cp}{^{14}Cs} \right)$$

Vs - volume of supernatant sample
^3Hp - ^3H counts in pellet
^3Hs - ^3H counts in supernatant
^{14}Cp - ^{14}C counts in pellet
^{14}Cs - ^{14}C counts in supernatant

(Also see Nicholls, 1974; Rottenberg, 1979; Halestrap and Quinlan, 1983). These values are expressed in μl/mg protein directly.

Radioactive Ion Distributions in the Determinations of Mitochondrial Membrane Potential

Methods essentially as described by Chappell and Crompton, 1979 were employed.

Mitochondria at 1 mg/ml were incubated at 30°C with shaking, as described above, except the radioactive isotopes were replaced by 2 μM [^3H] - $TPMP^+$ (1 μCi/ml) or 50 μM $^{86}Rb_2SO_4$ (0.2 μCi/ml) plus 0.1 μM Valinomycin (^{86}Rb samples were counted in the high energy ^{32}p-channel of the liquid scintillation counter). Membrane potential was calculated directly from the Nernst equation taking the counts/space equivalents when one isotope was employed.

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[X]in}{[X]out}$$

When dual label experiments were performed the spillovers of each isotope were calculated, and the counts used in the following manipulations corrected (cf. Rottenberg, 1979). Corrections were determined by counting single and mixed standards in the dual channel mode, as follows.

$$\text{Correction factor} = \frac{\text{Counts in } {}^{14}C\text{-channel due to } {}^{86}Rb}{\text{total counts in } {}^{14}C \text{ channel alone}}$$

Scintillation Cocktail

The scintillant employed provided universal counting facilities for dual channel counting, $^{3}H/\ ^{14}C$ or $^{14}C/\ ^{32}p$. 7g of butyl-PBD and 80g of naphthalene were dissolved in 600ml of toluene and 400ml of methoxy-ethanol.

Fluorometric Monitoring of Membrane Potential

Routinely 1mg/ml of mitochondria were incubated at 2 nmoles dye/mg protein in 250mM Sucrose, 10mM HEPES pH 7.5, 5mM succinate and 8 μM rotenone in 3ml four sided plastic cuvettes. Time dependent fluorescent readings were measured (Excitation 479nm-Emission 589nm) using a Perkin Elmer MPF 44 spectrofluorimeter and Hitachi recorder at 25°C. During calibration energised mitochondria were rapidly removed from the cuvette and the mitochondria separated by centrifugation, as described above, before counting the $[^{3}H]$ -$DSMP^{+}$ bound. All other experimental details are to be found in the legends of the figures.

Inhibitor Solutions

All inhibitors were serially diluted from stock ethanolic solutions, ethanol never to exceed 1% of the assay medium.

2.3. Results and Discussion

Mitochondrial Membrane Potential Dependent Fluorescence Responses with $DSMP^{+}$

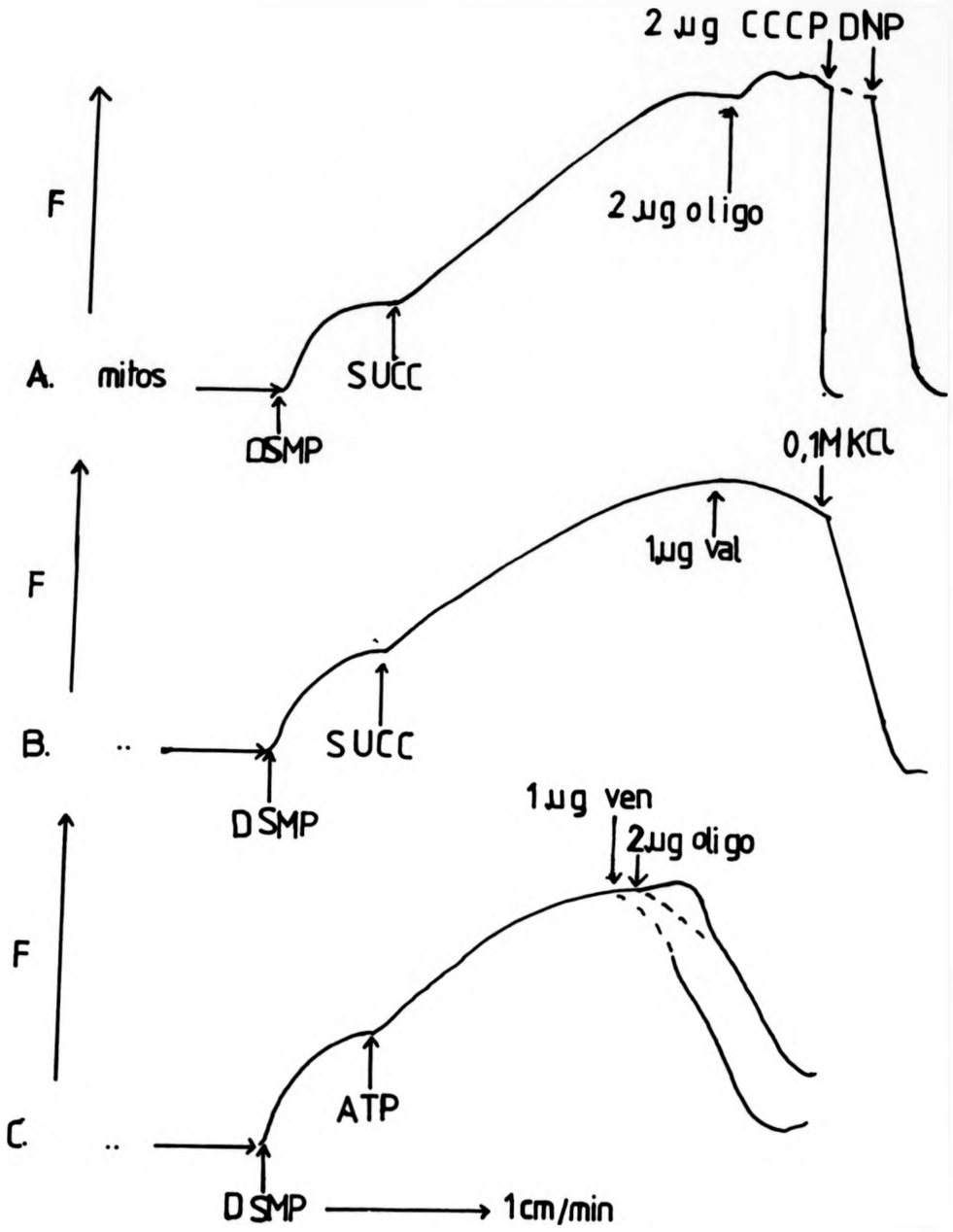
Figure 2.i presents a series of time dependent fluorescence traces of mitochondrial suspensions in the presence of the fluorescent dye $DSMP^{+}$. Changes in the emission of a single line, 589 nm (Excitation 479 nm), are maintained as the mitochondria undergo succinate driven respiration in the presence of rotenone to render complex I non-functional. The energisation of the mitochondrial membrane and development of a transmembrane potential is paralleled by the simultaneous

Figure 2.i

DSMP$^+$ Dependent Fluorescence Changes with Rat Liver Mitochondria

Legend

Mitochondria were suspended at 1mg/ml protein in 250mM Sucrose, 10mM HEPES pH 7.5, 8 µM Rotenone in 4ml four sided plastic cuvettes and placed in the fluorimeter (Ex 479 - Em 589nm, slits 5mm) before additions of 15µl 0.2mM DSMP$^+$ and either succinate to make 5mM or ATP to make 2.5mM. Inhibitor additions were made in ethanolic solutions as directed.



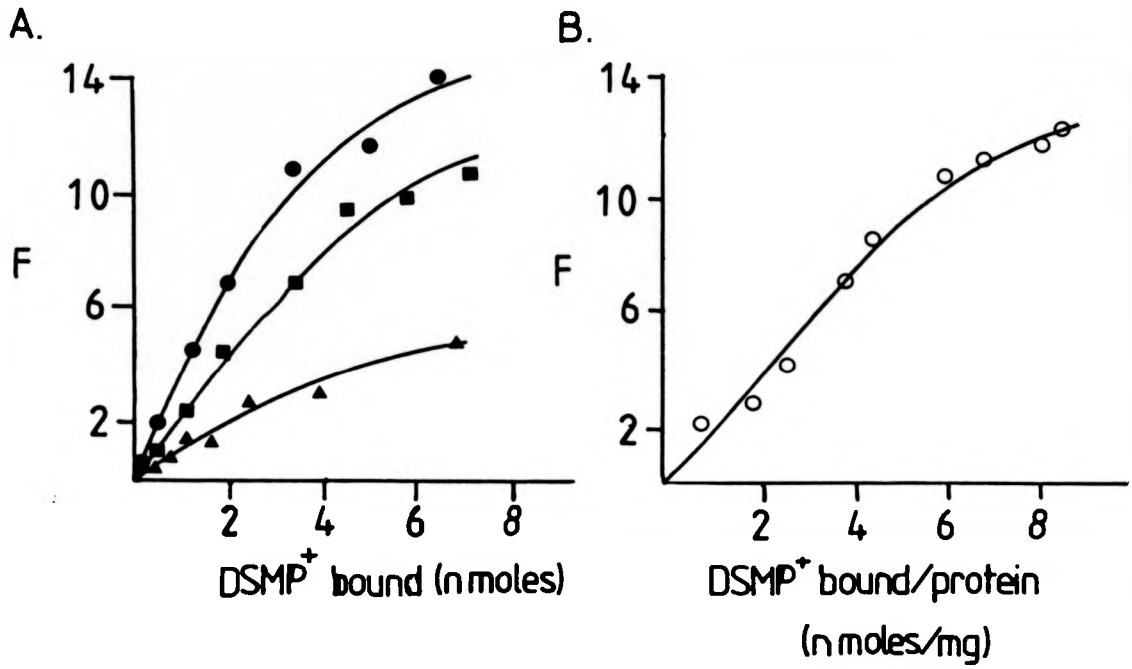
uptake of the lipophilic dye along the electrical gradient into a more apolar environment which results in a higher fluorescence intensity. Thus, the increase in fluorescence on addition of succinate monitors the establishment of a transmembrane potential via respiration, which may be circumvented by pre-incubation with antimycin A, thereby inhibiting complex III. The signal is uncoupler sensitive and the addition of CCCP or DNP result in sharp falls on the trace (see Figure 2.ia). In contrast, oligomycin produces a small increase in fluorescence (5-10%). Similar effects are achieved with the addition of potassium ions to mitochondria pre-incubated with valinomycin (see Figure 2.ib). Conversely, a transmembrane potential may be facilitated by the hydrolysis of ATP via the ATPase complex (V), which produces an increased signal, sensitive to uncouplers and inhibitors of the ATPase complex (see Figure 2.ic). The traces presented in Figure 2.i are consistent with the inhibitors' modes of action and the function of $DSMP^+$ fluorescence to monitor mitochondrial membrane potential in a time dependent manner.

Calibration of $DSMP^+$ Fluorescence

Calibration of $DSMP^+$ fluorescence and mitochondrial membrane potential was undertaken by measuring the fluorescence intensities of various dye/mitochondrial protein ratios and determining the bound dye by differential centrifugation of radio-labelled $DSMP^+$. Figure 2.iia presents the fluorescence intensity against the bound $DSMP^+$ for various fixed mitochondrial protein concentrations. These results are similar to those reported by Mewes and Rafael, (1981), indicating the relationship between fluorescence intensity and the $DSMP^+$ taken up by mitochondria approximates to linearity up to an intramitochondrial level of 3 nmoles dye/mg mitochondrial protein. Therefore, within the sensitivity limit of 0.1 nmoles dye/mg protein and the deviation from

Figure 2.ii

Plots of Fluorescence Intensity Against $DSMP^+$ Bound



Legend

A. Fluorescence intensity of $DSMP^+$ bound to rat liver mitochondria at (●) 1.8, (■) 0.9, (▲) 0.5 mg/ml protein with varying concentrations of [^{3}H]-$DSMP^+$ under energised conditions (see 'Materials and Methods').

B. Fluorescence intensity as a function of bound $DSMP^+$ per mg of mitochondrial protein.

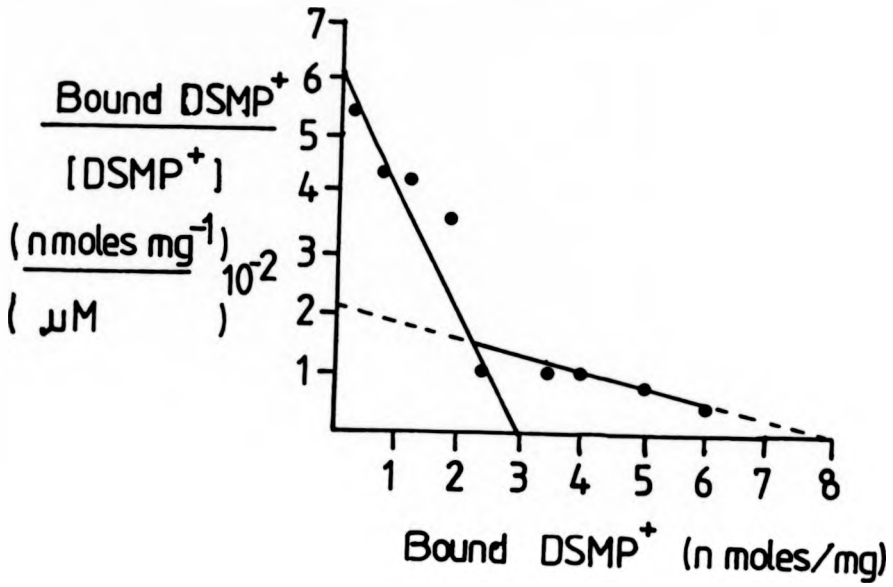
linearity at 3 nmoles dye/mg protein $DSMP^+$ fluorescence reporting accurately the uptake of the dye, as shown in Figure 2.iib. Further kinetic analysis performed by Mewes and Rafael, (1981), has shown that the uptake of $DSMP^+$ is strictly first order up to an intramitochondrial concentration of 4 nmoles/mg protein. This is strong evidence that the accumulation of $DSMP^+$ in mitochondria is solely due to permeation along the electrochemical gradient.

This conclusion can be further validated when one attempts to ascertain a binding constant for the association of $DSMP^+$ with rat liver mitochondria. The extent of accumulation dictating the binding constant and capacity are variable with energisation of the membrane. In practice, individual preparations of rat liver mitochondria at maximal stimulation of respiration (5 mM succinate in the presence of 8 μM rotenone) can produce a characteristic binding plot (see Figure 2.iii). Similarly uniform reductions in the membrane potential equate to varying dissociation constants. This experiment has been performed utilising a potassium diffusion potential in the presence of valinomycin to set up a controlled reduction in $\Delta\Psi$, the extent of which can be monitored by $DSMP^+$ fluorescence together with concentration dependent $[^3H]$-$DSMP^+$ binding and the standard isotope distribution techniques using $^{86}Rb^+$ and $[^3H]$-$TPMP^+$. Considering the upper limit of 3 nmoles dye/mg of protein on fluorescence linearity, the initial curve observed on the Scatchard plot for fully energised mitochondria fits well, with 3.2 nmoles dye/mg protein and an apparent K_D of 49 μM. The break in the Scatchard plot beyond 3 nmoles dye/mg bound may be indicative of the formation of $DSMP^+$. aggregates which may not penetrate the lipid bilayer of mitochondria, this is consistent with the fluorescence observations and those of Mewes and Rafael, (1981), (n=8 nmoles/mg and apparent K_D-355μM).

Figure 2.iv demonstrates the imposition of a potassium diffusion

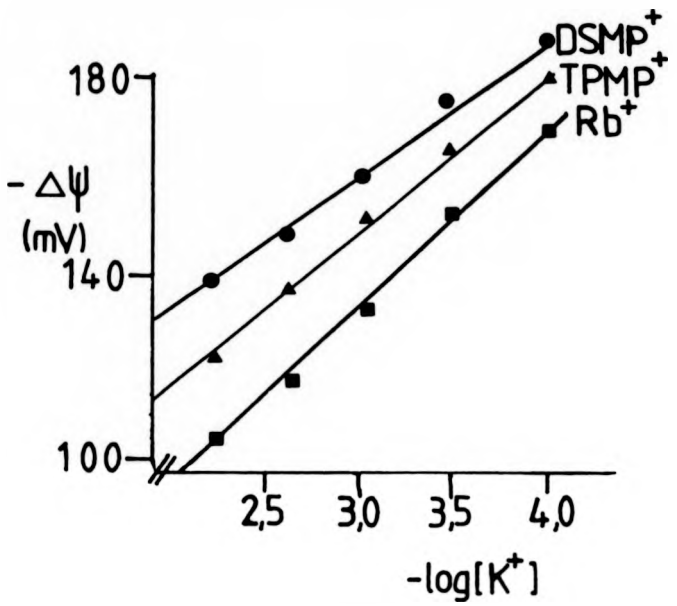
Figure 2.iii

Scatchard Analysis of $DSMP^+$ Binding to Energised Mitochondria



Legend:
Mitochondria (0.9mg) were incubated under energised conditions with varying concentrations of [^{3}H]-DSMP I before rapid separation of mitochondria and supernatant as described in 'materials and methods'. The supernatant count was equated to the free ligand and the pellet count to the bound dye. The superimposed curves are the products of two regression analyses and the intercepts calculated accordingly.

Figure 2.iv



Reduction in Mitochondrial Membrane Potential by Valinomycin Mediated Potassium Uptake

Legend:

0.9mg/ml mitochondrial protein was incubated as described in 'materials and methods' with 0.4 µM valinomycin and the relevant radioactive ion, 50mM $^{86}Rb^{+}$ (0.2µCi/ml), 2µM [^{3}H]-$TPMP^{+}$ (1µCi/ml) and 1µM [^{3}H]-$DSMP^{+}$ (0.2 µCi/ml) and subjected to various KCl concentrations prior to removing 1ml aliquots for centrifugation, as described in 'materials and methods' (matrix volume taken as 0.59µl/mg, as determined in 'materials and methods').

potential on rat liver mitochondria, the reduction in $\Delta\Psi$ is measured by radioactive distribution techniques using $[^3H]$-DSMP-I, $[^3H]$-TPMP Br and $^{86}Rb_2SO_4$. The collapse of membrane potential against the diffusion gradient is linear when plotted against negative log $[K^+]$ in all cases; further evidence of good calibration. These results are, in general, in agreement with those of Mewes and Rafael, (1981). It is also noted as observed by the previous authors that the methods are not equivalent at high potassium ion concentrations, although the discrepancies observed here are not so large, the over estimation of $^{86}Rb^+$ uptake when $[K^+]$ is in the millimolar range may well be due to energy driven parallel proton extrusion.

Determining the apparent dissociation constant at various extrapolated values of $\Delta\Psi$, one observes a decline in their binding affinities as a logarithmic function. Plot 2.v represents the relationship between the apparent K_D and the imposed potential, where proportional fluorescent changes are observed (ΔF) below 3 nmoles/mg protein binding of $DSMP^+$.

$$\Delta\Psi = \log (cK_D) = \log (c_1 \Delta F)$$

(c and c_1 are constants)

It must be noted further that the proportionality constants relating these terms must remain to be determined experimentally since systematic variation will occur, particularly when using high ionic strength solutions with diffusion gradients.

Robertson and Rottenberg (1983), have derived the following relationship between fluorescence probe relative binding and surface potential ($\Delta\Psi s$).

$$K_D = \exp\ (\ G^o/RT)\ \exp\ (ZF\Delta\Psi s/RT)$$

Where Z is the charge of the ion and G^o true Gibbs free energy, since

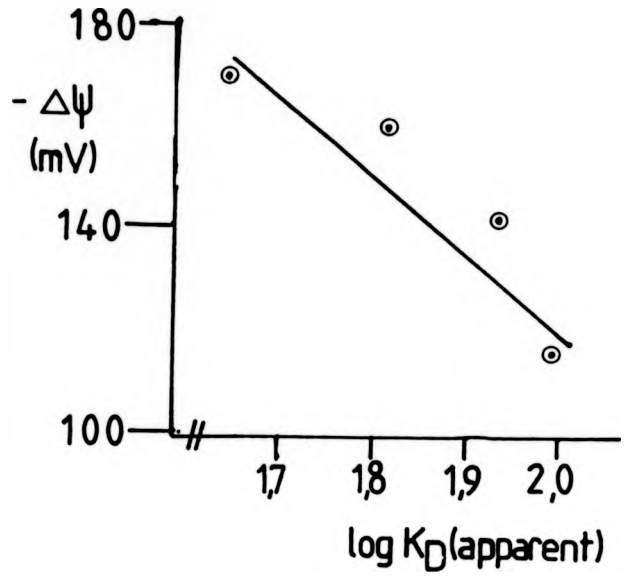
$$K_D{}^o = \exp\ (\Delta G^o/RT)$$

$$K_D/K_D{}^o = \exp\ (Z\Delta\Psi s/RT)$$

$$\Delta\Psi s = (RT/ZF)\ \ln\ (K_D/K_D{}^o)$$

Figure 2.v

Relationship of K_D (apparent) and $\Delta\Psi$ for $DSMP^+$



Legend:
The points were generated from individual Scatchard plots varying concentrations of potassium ions corresponding to known values of membrane potential calculated from the Nernst equation with with $[^3H]$-$DSMP^+$ (see Figure 2.iii). The straight line plot is the result of regression analysis.

In the case of a probe obeying the Nernst equation and avoiding surface stacking effects, the actual dissociation constant (K_D^o) should tend to unity where the observed potential becomes directly related to the log of the apparent dissociation constant, under similarly energised conditions. Validation of this relationship occurs in the case of $DSMP^+$ as the monitoring permeant cation (see Figure 2.v), providing an intramitochondrial dye concentration of 3 nmoles/mg protein is not exceeded. Therefore, with defined fluorescence intensities per nmole $DSMP^+$ bound, the mitochondrial membrane potential may be determined accurately. The following relationship has been defined by Mewes and Rafael (1981) for such determinations.

$$\Delta\Psi = -RT/F \ln \frac{[DSMP^+] \text{ in}}{[DSMP^+] \text{ out}}$$

$$[DSMP^+]in = \frac{F_I}{F_D - Vi}$$

$$[DSMP^+] \text{ out} = \frac{[DSMP^+] \text{ total} - [DSMP^+] \text{ in. } Vi}{V - Vi}$$

Where F_I and F_D equal the fluorescence intensity and intensity per nmole $DSMP^+$ bound respectively, V the total incubate volume and Vi the matrix volume.

However, Mewes and Rafael (1981), have pointed out in practice that circumstantial definition of the fluorescence intensity/nmole $DSMP^+$ bound (F_D) is not required if the organelles under investigation are capable of maximal energisation, such as rat liver mitochondria. Under state 4 conditions, the fluorescence may be equated with the theoretical uptake of dye from an assumed membrane potential of -190mV and the measured matrix volume. Further extrapolations of various membrane potentials may be made with observed fluorescence intensities based on published data. However, the present study routinely estimates membrane potential at -180mV and fluctuations have been observed,

particularly when mitochondria continue to undergo fatty acid metabolism. The addition of bovine serum albumin will limit this problem.

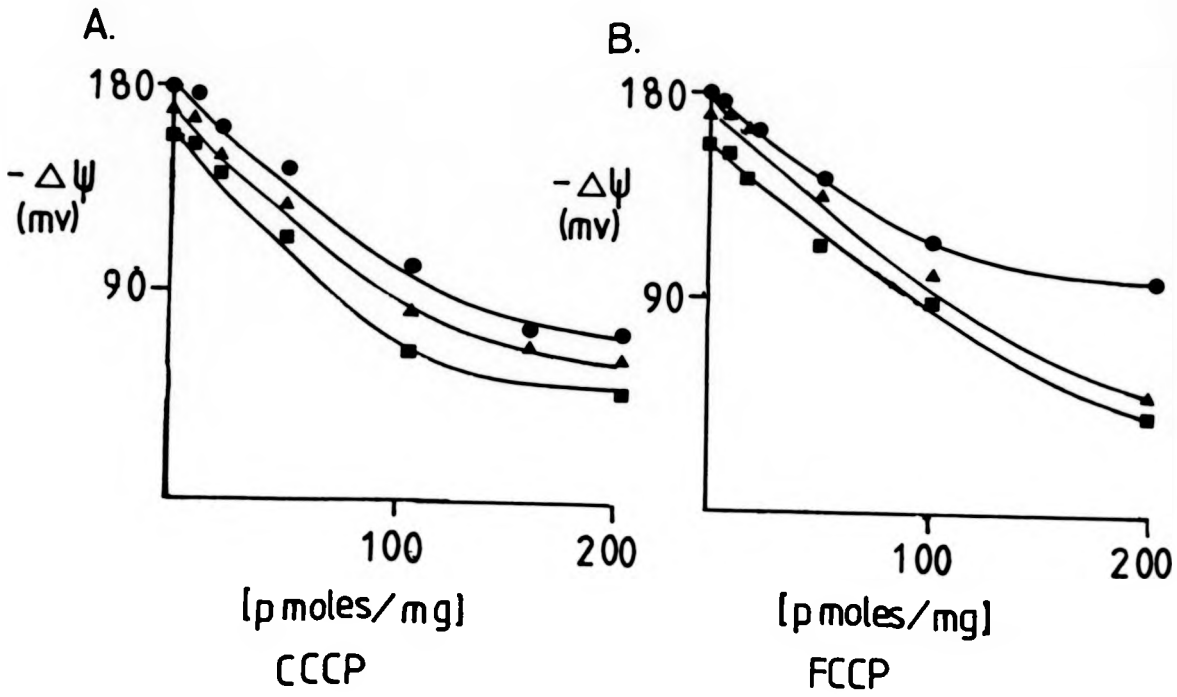
The values of mitochondrial membrane potential presented here, -140 to 180mV dependent on the method of estimation, are all sufficiently high with moderate pH gradients to drive ATP synthesis as predicted by the chemiosmotic hypothesis. However, the values are far in excess of those measured by Tedeschi and colleagues with microelectrodes and giant mitochondria (Compo et al, 1984a). Furthermore, they have shown that the impaled giant mitochondria maintain only a small pH gradient by the injection of pyranine, a pH sensitive fluorescent probe, but still undergo succinate driven ATP synthesis (Compo et al, 1984a,b). Therefore, the giant mitochondria appear to drive oxidative phosphorylation in the absence of a functional proton motive force, as ascertained by the microelectrodic determination of $\Delta\Psi$ and pyranine fluorescence for ΔpH, a conclusion which is clearly at odds with the ion distribution data and the chemiosmotic hypothesis. Tedeschi has argued that the ion distribution techniques are prone to large over-estimates of the accumulation of permeant labelled ions and dyes. However, based on similar experiments to those discussed above with thylakoid membranes, Compo et al, (1985) have demonstrated a large ΔpH component which they conclude as functional in photophosphorylation.

Uncoupler Titrations of Mitochondrial Membrane Potential

Further validation of the fluorescence method was made by comparing the titrations of potent protonophores with those produced by the ion distribution techniques. Fig 2.vi shows the titration curves for the uncouplers CCCP(A) and FCCP(B) by various methods. The techniques employed are in basic agreement as to the actions and general potency of the uncouplers, but again discrepancies are observed between the fluorescence technique and the ion distribution methods. $^{86}Rb^{+}$

Figure 2.vi

Uncoupler Titrations of Membrane Potential



Legend:

A. Titration with CCCP; B. Titration with FCCP.
1mg/ml mitochondrial protein was incubated as described in 'materials and methods' with (■) 25mM $^{86}Rb_2SO_4$ (0.2 μCi/ml) (plus 0.2μM valinomycin), (▲) 2μM [^{3}H]-$TPMP^+$ (1 μCi/ml), before separation by centrifugation and sampling. 10 μM $DSMP^+$ (●) was utilised in the fluorescence assay. Matrix volumes were taken as 0.59μl/mg in sucrose media.

uptake consistently underestimates the membrane potential as ascertained by $DSMP^+$ fluorescence and $[^3H]$ $TPMP^+$ uptake, equilibrium of the rubidium cation via valinomycin carriage may not be as responsive as the direct diffusion of lipophilic cations. However, as soluble compounds of the membrane, the previous cations are prone to binding errors, although corrections are made for this in the case of $DSMP^+$ during calibration. The underestimation of the ion distribution techniques may also have a systematic basis, since the mitochondrial suspensions are prone to anaerobiosis on centrifugation, together with the accumulation of inhibitor in the mitochondrial pellet.

The fluorescence determination of membrane potential provides a quick responsive time dependent assay which may be used for kinetic experiments. The initial rate of depolarisation of $\Delta\Psi$ after the introduction of an uncoupler can be monitored as membrane potential dependent fluorescence reduction, which should be indicative of the rate of proton equilibrium across the membrane facilitated by the uncoupler. The concomitant equilibration of membrane potential on titration may then be the result of an equilibrium between the proton output via respiration and the carrying capacity of the translocator, which should be a function of its diffusion rate in the bilayer. However, respiration driven proton pumping does not have to proceed at a fixed rate, and resulting equilibrium stability may reflect this.

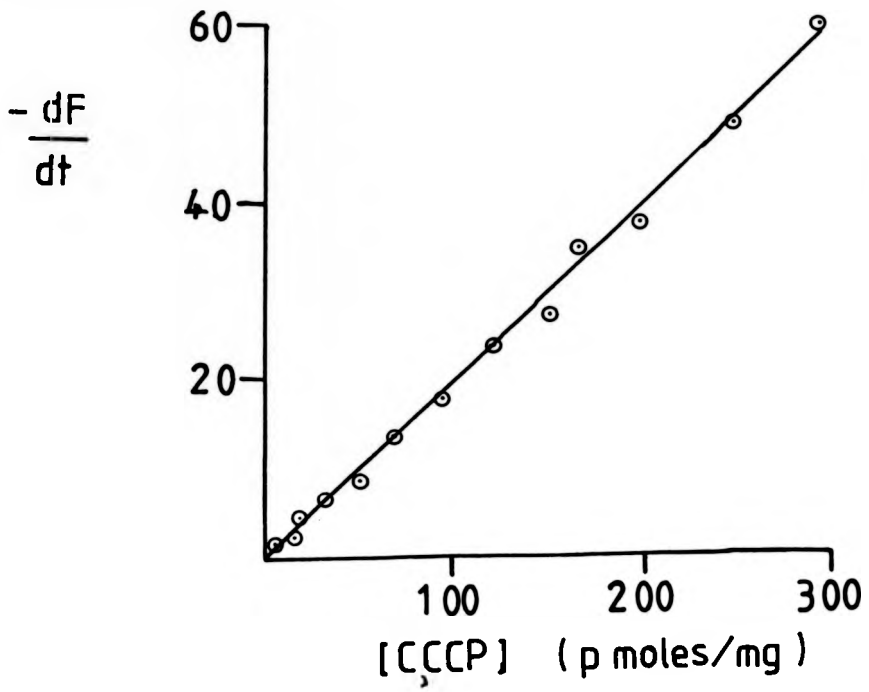
The observed change in the initial rate of time dependent fluorescence reduction is apparently directly proportional to the imposed uncoupler concentration, in the case of functional amounts of CCCP and FCCP. Figure 2vii demonstrates the linear relationship between fluorescence changes and CCCP concentration where the curve approaches the origin.

$$\frac{-dF}{dt} = [CCCP]$$

This relationship is consistent with CCCP functionally acting purely

Figure V.ii

Time Dependent Fluorescent Changes of $DSMP^+$ Against CCCP Concentration with Respiring Mitochondria



Legend:
1mg/ml mitochondria were incubated as described in 'materials and methods' before the addition of various titres of uncoupler at peak fluorescence. The resulting changes were recorded as a function of time via a chart recorder linked to the fluorimeter.

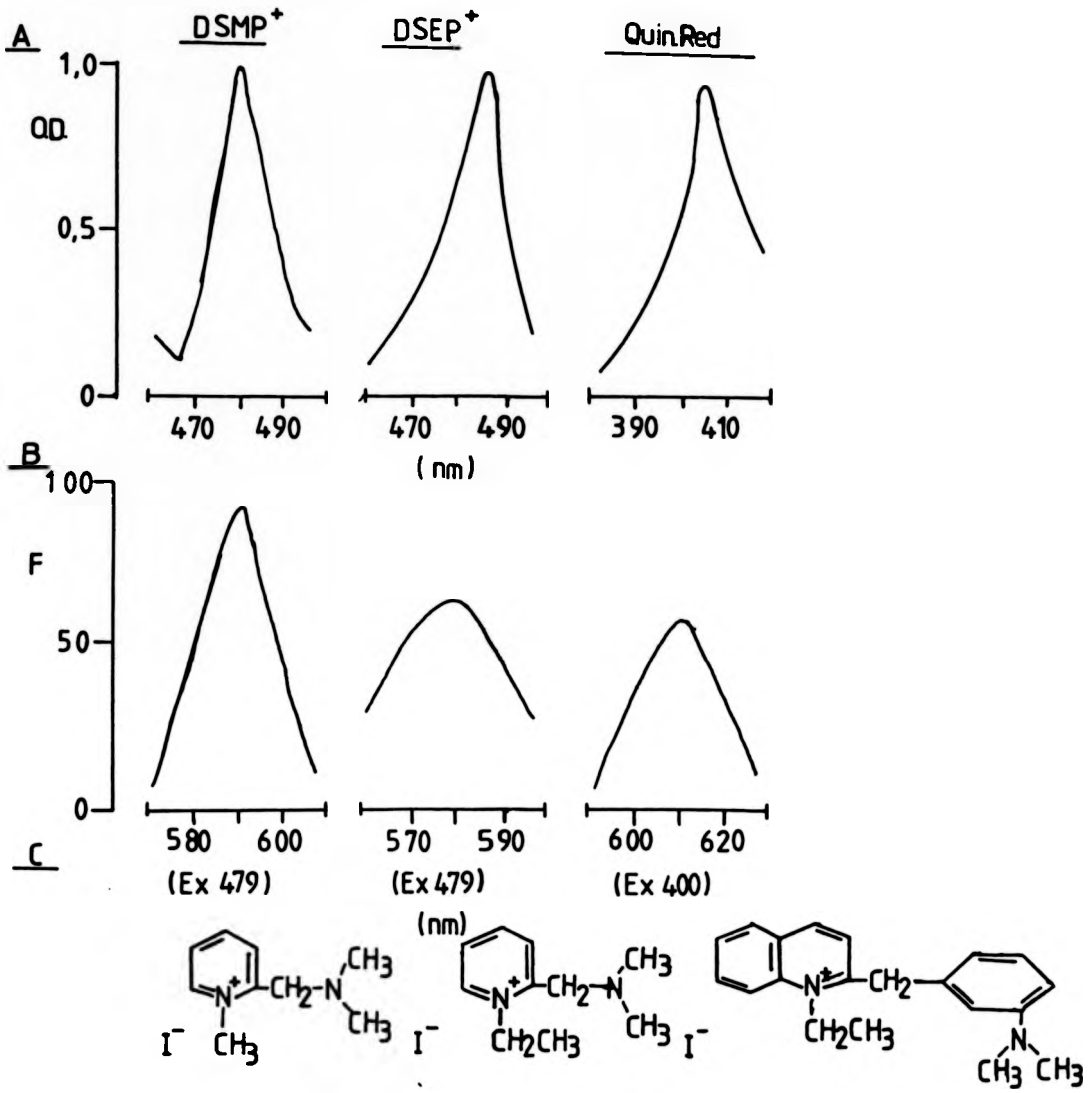
in a charge carrying fashion rather than via interactions with the membrane or its components. Conversely, $DSMP^+$ is functioning within the confines of the Nernst equation.

The effects of various inhibitors of the mitochondrial H^+-ATPase were tested in the $DSMP^+$ based system (see Figure 2.i for initial experiments). Interestingly, low titres of oligomycin and venturicidin (1.0 µg/mg and 0.6µg/mg respectively) produced increases in the membrane potential of approximately -15 to -20mV. These effects are consistent with the inhibitors modes of action, preventing a back flow of protons via the ATPase. Further titrations produced small reductions in the membrane potential to -160mV at >10µg/mg. The inhibitor ossamycin had no significant effect on membrane potential, which, although plausible bearing in mind its function as an ATP synthase inhibitor, is inconsistent with a recent report on E.coli, where it apparently acts as an uncoupler (Perlin et al, 1985), albeit at high concentrations (>40µg/mg).

Fluorescence Analogues of $DSMP^+$

The $DSMP^+$ analogues $DSEP^+$ and Quinaldine Red were also tested as fluorescence indicators of mitochondrial membrane potential. Figure 2.viii presents the relative absorption and fluorescence maxima employed in mitochondrial experiments. Quinaldine Red produced a smaller relative fluorescence signal which proved to be insensitive to changes in mitochondrial membrane potential (Ex(max) 400nm - Em(max) 610nm). However, $DSEP^+$ provided a comparably strong emission to that of $DSMP^+$ which also responded in higher fluorescent yields in the presence of hydrophobic membranes (Ex(max) 479nm - Em(max) 582nm). As presented in Figure 2ix, mitochondrial preparations showed energisation specific fluorescence similar to $DSMP^+$ with the advantage of a lower basal fluorescence. However, $DSEP^+$ produces a relatively smaller signal and as a consequence, does not provide the same sensitivity as $DSMP^+$.

Figure 2.viii



Spectroscopic Properties of $DSMP^+$, $DSEP^+$ and Quinaldine Red

Legend:

A. Absorption spectra of $DSMP^+$, $DSEP^+$ and Quinaldine Red (0.5mM in 50% ethanol, slit width 10nm)

B. Fluorescence spectra of $DSMP^+$, $DSEP^+$ and Quinaldine Red

C. Chemical structures of $DSMP^+$, $DSEP^+$ and Quinaldine Red

Figure 2.ix

Comparison of the Fluorescent Responses of $DSMP^+$ and $DSEP^+$ on Mitochondrial Energisation

Legend:
Mitochondria were incubated at 0.9mg/ml in 250mM sucrose, 10mM HEPES pH 7.5, 8 µM Rotenone in 4ml four sided plastic cuvettes and placed in the fluorimeter (A.DSEP Ex 479 → Em 582nm, B.DSMP Ex 479 → Em 589nm) before additions of 15 µl 0.2mM dye and succinate to make 5mM with rapid stirring. Inhibitor additions were made in ethanolic solution.

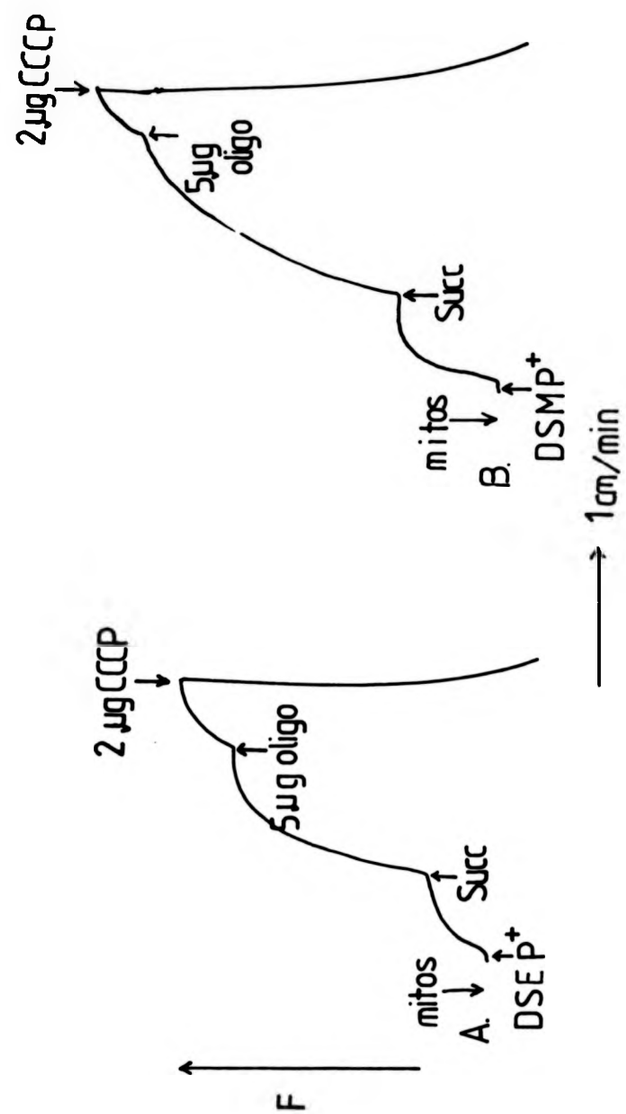


Figure 2.ix

$DSEP^+$ behaved similarly to $DSMP^+$ in all other aspects, producing similar inhibitor response traces and uncoupler titration curves. Although the precise calibration of the $DSEP^+$ response in mitochondria could not be attempted without a radioactive analogue, the compound provided a useful probe of mitochondrial membrane potential. Experiments will be described in Chapter 6 that utilise the compounds low non specific fluorescence to monitor *in situ* protoplast lysates of yeast for mitochondrial dependent potential changes. Similarly, $DSEP^+$ may have some potential to monitor mitochondrial membrane potential changes in whole cells, similar to the studies of Rafael and Nicholls, (1984), with brown adipose tissue and $DSMP^+$.

2.4 Conclusion

$DSMP^+$ has been shown to produce increased fluorescent yields in the presence of energised mitochondrial membranes, either respiration driven or by ATP. The fluorescence signal is sensitive to uncouplers which titrate the signal in a concentration dependent manner. The increased fluorescence signal has been shown to be dependent on the intramitochondrial $DSMP^+$ level by the uptake of $[^3H]$-$DSMP^+$ which produces a linear relationship up to 3 nmoles dye/mg protein. Further, the uptake of the dye occurs in response to the imposed potential giving rise to a series of apparent dissociation constants related in a linear log fashion, as predicted by the Nernst equation.

Membrane potentials calculated from $DSMP^+$ fluorescence have consistently yielded higher estimates than those based upon the isotopic distributions of $[^3H]$-$TPMP^+$ and $^{86}Rb^+$ in the presence of valinomycin. However, all these techniques produce similar titration trends with valinomycin mediated potassium uptake and uncoupling. The membrane potentials measured by the above methods are all sufficiently high enough to drive ATP synthesis in coupled mitochondria, as predicted by the

chemiosmotic hypothesis.

The $DSMP^+$ analogue $DSEP^+$ has also been shown to produce membrane potential dependent fluorescence responses with isolated rat liver mitochondria.

CHAPTER 3

The Effects of Trialkyltins on the Energetic Functions of Rat Liver Mitochondria

3.1 Introduction

Trialkyltin compounds have been shown to be potent inhibitors of mitochondrial oxidative phosphorylation, oligomycin sensitive adenosine triphosphatase (OS-ATPase) and some energy linked reactions eg. ^{32}Pi-ATP exchange and transhydrogenase (Aldridge and Street, 1964; Aldridge and Rose, 1969 , Sone and Hagihara, 1964; Emanuel et al, 1984). These effects have been attributed to three basic modes of action. The first is a direct inhibition of the F_1 Fo-ATPase complex which manifests itself in the loss of both ATP hydrolytic and ATP synthetic activities (Aldridge and Street, 1964; Stockdale et al, 1970; Rose and Aldridge, 1972). This 'oligomycin-like effect' appears to be the result of an interaction with the Fo component of the F_1Fo-ATPase (Sone and Hagihara, 1964; Stockdale et al, 1970) causing the inhibition of H^+ conduction through the complex (Gould, 1978; Dawson and Selwyn, 1975; Papa et al, 1982).

The second mode of action is to induce a chloride/hydroxyl exchange activity across the inner mitochondrial membrane, which facilitates an equilibrium of respiratory generated pH difference resulting in general uncoupling (Selwyn et al, 1970; Dawson and Selwyn, 1974).

The third reported activity is that of a gross swelling of the mitochondria in either potassium chloride or potassium isothionate media, causing loss of energetic function (Aldridge and Street, 1964; Aldridge et al, 1977).

Studies on the nature of the trialkyltin binding site(s) of submitochondrial particles from rat liver, beef heart and yeast, have yielded complex binding curves which have been interpreted as indicative of

at least two classes of binding site (Aldridge and Street, 1970; Dawson and Selwyn, 1975; Cain and Griffiths, 1977). The high affinity component which is consistent with the loss of ATPase activity (Rose and Aldridge, 1972; Cain and Griffiths, 1977; Farrow and Dawson, 1978), and a low affinity component, the function of which has been questioned (Farrow and Dawson, 1978).

The chemical nature of the triorganotin binding site has been studied on the basis of organotin interactions with various proteins, and claims that histidine and thiol or dithiol groups are involved have.been made (Rose, 1969; Elliot and Aldridge, 1977; Elliot et al, 1979; Gould, 1978; Dawson et al, 1982). Functional indications as to the nature of trialkyltin binding have come from experiments where mono- and dithiols have been used to specifically reverse the inhibitory activities of trialkyltins towards ATP synthesis in submitochondrial particles (Cain et al, 1977; Emanuel et al, 1984; Yagi and Hatefi, 1984). It has been suggested from such experiments that dithiols may play an important role in the conduction of protons through the Fo moiety of the mitochondrial ATPase complex.

The present chapter reports the activities of various trialkyltins on the ATP synthetic and hydrolytic enzyme functions of rat liver mitochondria, together with estimates of the parameters of proton motive force (ΔP) during inhibition. Evidence will be presented that trialkyltins may act as potent 'oligomycin-like' inhibitors and also have the capacity to uncouple, independent of the phenomenon of chloride/hydroxyl exchange. The relative trialkyltin concentrations in which these events take place are in the order of ATPase > ATP synthase $\geq \Delta p$ reduction.

3.2 Materials and Methods

Materials

All chemicals used were of analytical grade. All trialkyltin chlorides

were purchased from BDH Chemicals Ltd., UK, except trioctyltinchloride which, with tricylchlohexyltin hydroxide was obtained from Dow Chemical Company USA. Tributyltin oxide, tributyltin phosphate, tributyltin malate and tributyltin acetate were gifts from Schering Industrie-Chemikalien, West Germany.

Triethyltin sulphate was made in this laboratory from the hydroxide donated by the Tin Research Institute. Dibutylchloromethyltin chloride was synthesised in this laboratory by Dr. D.E. Griffiths. Yeast hexokinase (type F-300), glucose and lactate were purchased from Sigma Chemical Company. ADP and ATP were purchased from Boehringer Corporation Ltd. Other special reagents and inhibitors were obtained as listed in Chapter 2 of this thesis.

Methods

Rat liver mitochondria were prepared as described in Chapter 2. Similarly, the techniques for measuring membrane potential and mitochondrial volumes were also described in Chapter 2.

ATP Synthesis

Oxidative phosphorylation was assayed in a glucose-hexokinase trap system, containing suspension buffer (250mM Sucrose, 10mM HEPES pH 7.5) plus 20mM glucose, 5mM potassium phosphate, 2mM Mg SO_4, 2mM ADP, 5mM succinate and 5 units of yeast hexokinase. Mitochondria were pre-incubated with inhibitors for 5 minutes on ice, prior to initiation of the assay at $30^{o}C$ in a shaking water bath.

The assay was run for 30 minutes in open 20ml phosphorylation pots to ensure adequate aeration, in a volume of 1ml. The reaction was terminated with 1ml of 10% trichloroacetic acid. The protein precipitate was then removed by centrifugation in a bench centrifuge, before 0.1ml aliquots were removed for phosphate determination. ATP synthesis was measured as the disappearance of inorganic phosphate from the medium.

ATP Hydrolysis

ATPase activity was determined by measuring the release of inorganic phosphate liberated from 5mM ATP. 1ml assays containing 0.2-0.5mg.of mitochondrial protein were preincubated in phosphorylation buffer minus hexokinase, glucose and ADP with or without inhibitors at $0^{o}C$ for 5 minutes. The reaction was then initiated with ATP at $30^{o}C$. Assays were terminated after 10-15 minutes with 1ml of 10% trichloracetic acid. The coagulated protein was removed by centrifugation in a bench centrifuge before removing a 0.5ml aliquot for phosphate determination.

Estimation of ΔpH

The pH gradient between mitochondria and the medium was established using the distribution of $[^{14}C]$-lactate, a permeant conjugate acid, as described by Chappell and Crompton, 1979). Briefly, the pH gradient was determined in the presence of $[^{3}H]$-TPMP or ^{86}Rb while simultaneously measuring the membrane potential in 4ml incubations containing 1mg/ml mitochondria, as described in Chapter 2. 1ml aliquots of the incubation medium was removed and the mitochondria rapidly separated by centrifugation for 2 minutes in an Eppendorf microcentrifuge. 0.1ml of the supernatant was taken for the external count and the pellet resuspended and acidified with 0.25ml of 10% perchloric acid, before repelleting the mitochondria and taking 0.1ml of the supernatant for counting. The samples were mixed with scintillant and dual channel counted in a Packard Tricarb Scintillation Counter.

Estimation of Inorganic Phosphate

Phosphate was determined by the methods of Fiske and SubbaRow, 1925, and Heinonen and Lahti, 1980. Both methods depend on the formation of a phosphomolybdate complex, the earlier method relying upon reduction with 1-amino-2-napthol-4-sulphonic acid to form molybdenum blue, which is measured spectrophotometrically at 690nm against phosphate

standards. The latter method utilises the yellow chromophore of phosphomolybdate when dissolved in acetone, to monitor phosphate concentrations against standards at 355nm.

Fiske and SubbaRow Assay

Inorganic phosphate (10-500 nmoles) in 0.5ml H_2O was added to a 4ml plastic test tube containing 1.5ml water and 0.3ml 2.5% amonium molybdate in 5M H_2SO_4. The contents were vortexed and 0.2ml of a 0.2% 1-amino-2-napthol-4-sulphonic acid; 12% $NaHSO_3$; 2.4% Na_2SO_3. $7H_2O$ solution (ANSA reagent) was added. The tube was vortexed once more and allowed to stand for 20 minutes before determining the optical density against a reagent blank at 690nm. The phosphate content of the sample was then obtained from a standard curve established under similar conditions.

Heinonen and Lahti Assay

Inorganic phosphate (50-1500 nmoles) in 0.5ml H_2O was added to 6ml plastic tubes containing 4ml of a solution of 1:1:2 volumes of 10mM ammonium molybdate ($(NH_4)_6 Mo_7 O_{24}.4H_2O$), 5M H_2SO_4 and acetone respectively (AAM solution). The contents were vortexed and 0.4ml of 1M citric acid was added before vortexing once more, and reading immediately at 355nm against a reagent blank. Again the phosphate content of the sample was then obtained from a standard curve established under similar conditions.

Trialkyltin Solutions

All trialkyltins were added to the assays as ethanolic solutions serially diluted from 10mM stock.

3.3 Results

Mitochondrial Volume

It has been proposed by Aldridge *et al*, 1977, that chloride dependant stimulation of ATP hydrolysis in rat liver mitochondria is related to small scale mitochondrial swelling, which precedes gross swelling

as the concentration of triorganotin is increased. To understand these effects and any possible effects on membrane potential determination, the mitochondrial volumes of progressively inhibited samples were measured under similar conditions to those employed for the determination of ATP hydrolysis activity (cf. 'Materials and Methods'), in the presence and absence of 0.15M NaCl. These results are presented in Tables 3i and 3ii for [^{14}C]-sucrose and [^{14}C]-mannitol respectively.

As discussed earlier in Chapter 2 of this thesis, internal mitochondrial space, as determined using [^{14}C]-mannitol over [^{14}C]- sucrose produces slightly different results. Internal space determined in the presence of mannitol, consistently gives smaller volumes although the magnitude of the decrease varies over a greater range (0.08-0.17µl/mg) under the influence of trialkyltin mediated swelling. This variation has been observed previously by Halestrap and Quinlan, 1983, although they reported typical values of 0.46 µl/mg and 1.68 µl/mg for mannitol and sucrose respectively, under non-energised conditions. As pointed out by the above authors, this observation has an important consequence for the calculation of permeant ion concentrations within the mitochondrial matrix and ultimately the calculations of the parameters of proton motive force. However, the difference in matrix volume observed here are not so great, and will make a maximal difference of 12mV to Δp.

A chloride dependent ATPase activity was observed with triethyltin sulphate, at concentrations ranging between 0.25 and 1.00 nmoles/mg of mitochondrial protein. Within the range the mitochondrial volume appears to increase steadily before determinations became variable, presumably as the integrity of the membrane is disrupted. These phenomena presumably result from chloride/hydroxyl exchange as they are not observed in minimal halide media (under these conditions the

Table 3.i

The Effect of Triethyltin Sulphate on Mitochondrial ATPase Activity and Volume

Triethyltin Sulphate (nmoles/mg protein)	Mitochondrial Volume (µl/mg protein)		Mitochondrial ATPase (nmoles/mg/minute)	
	0.15M NaCl		0.15M NaCl	
	-	+	-	+
0	0.70	0.68	32	32
0.25	0.71	0.78	30	42
0.50	0.71	0.97	25	49
1.00	0.70	1.20	22	38
2.00	0.76	1.98	18	23
5.00	0.77	-	12	18
10.00	0.85	-	2	4
15.00	0.96	-	2	2
20.00	1.65	-	2	2

Legend:

These determinations of mitochondrial volumes are the averages of triplicates using $[^{14}C]$-sucrose and $[^{3}H]$-H_2O. Mitochondria were incubated with various titres of triethyltin on ice before dilution into the assay media at 4mg/ml and 0.2mg/ml for volumetric and ATPase assays respectively.

Table 3.ii

The effect of Triethyltin Sulphate on Mitochondrial ATPase Activity and Volume

Triethyltin Sulphate (nmoles/mg protein)	Mitochondrial Volume (µl/mg protein)		Mitochondrial ATPase (nmoles/mg/minute)	
	0.15M NaCl		0.15M NaCl	
	-	+	-	+
0	0.59	0.60	34	34
0.25	0.60	0.68	32	39
0.50	0.64	0.86	30	50
1.00	0.62	1.09	29	43
2.00	0.68	1.83	27	29
5.00	0.73	-	15	18
10.00	0.78	-	3	5
15.00	0.85	-	2	4
20.00	1.48	-	2	2

Legend:

Again these mitochondrial volume determinations were made in triplicate under similar conditions to those described in the legend to Table 3.i, the exception being [^{14}C]-mannitol replaced [^{14}C]-sucrose.

concentration of chloride in the media can never exceed 0.01mM). These results are in agreement with the functions proposed by Aldridge et al, 1977, for mitochondria suspended in chloride containing media. The mitochondria suspended in minimal halide did not swell at such low concentrations of triethyltin sulphate, but did increase rapidly in volume at 15 nmoles/mg. Further additions indeed appeared to perturb the integrity of the membrane in a non-specific manner. Concentrations of trialkyltin over 25 nmoles/mg have been previously reported to cause gross swelling and release of soluble protein, suggesting that trialkyltins induce lysis and/or dissociation of structural elements of the mitochondrial membrane (Wulf and Byrington, 1975). The prediction that high concentrations of triorganotins cause gross perturbations of the integrity of the mitochondrial membrane is consistent with the above findings. The concomitant increase in ATPase activity following large scale swelling reported for trimethyltin by Aldridge et al, 1977, was not observed in these experiments, presumably due to the enhanced 'oligomycin-like' activity of triethyltin over that of trimethyltin.

It is clear from these results that concentrations of triorganotins less than 20 nmoles/mg under minimal halide conditions should not perturb the integrity of the mitochondrial membrane unduly.

The Inhibition of ATP Synthase and Hydrolase Activities

The results presented in Table 3iii confirm that trialkyltins are potent inhibitors of oxidative phosphorylation and ATP hydrolysis in rat liver mitochondria. The sensitivities of these reactions under minimal halide conditions, as presented as I_{50} values in Table 3iii, represent the abilities of various trialkyltins to apparently inhibit the mitochondrial ATPase complex. The most active compound presented here is dibutylchloromethyltin chloride (DBCT), which is equally the

Table 3iii

The Effects of Trialkyltins on Mitochondrial ATPase and ATP synthase of Rat Liver

Trialkyltin		pI_{50} (nmoles/mg protein)±SD	
		ATPase	ATP synthase
Trimethyltin chloride	(2)	>100	>100
Triethyltin chloride	(5)	2.8 ±0.4	10.0 ±0.5
Triethyltin sulphate	(5)	2.8 ±0.4	9.8 ±0.5
Tripropyltin chloride	(4)	1.8 ±0.2	7.6 ±0.5
Tributyltin chloride	(6)	2.0 ±0.2	7.8 ±0.2
Tributyltin oxide	(3)	1.8 ±0.2	7.6 ±0.4
Tributyltin acetate	(3)	2.0 ±0.2	7.8 ±0.2
Tributyltin phosphate	(3)	2.5 ±0.5	8.0 ±0.5
Tributyltin malate	(3)	2.5 ±0.5	8.0 ±0.5
Dibutylchloromethyltin chloride	(6)	1.0 ±0.2	2.5 ±0.4
Trioctyltin chloride	(2)	5.0 ±0.4	14.5 ±1.0
Tricyclohexyltin hydroxide	(2)	3.0 ±0.4	9.0 ±0.4
Triphenyltin chloride	(6)	1.8 ±0.2	7.8 ±0.4

Legend:

ATPase and succinate driven ATP synthase activities were estimated as described in 'materials and methods'. The numbers in parenthesis correspond to the numbers of separate duplicate experiments. Control ATPase and ATP synthase rates ranged between 30-40 nmoles/mg minute and 80-100 nmoles/mg/minute respectively.

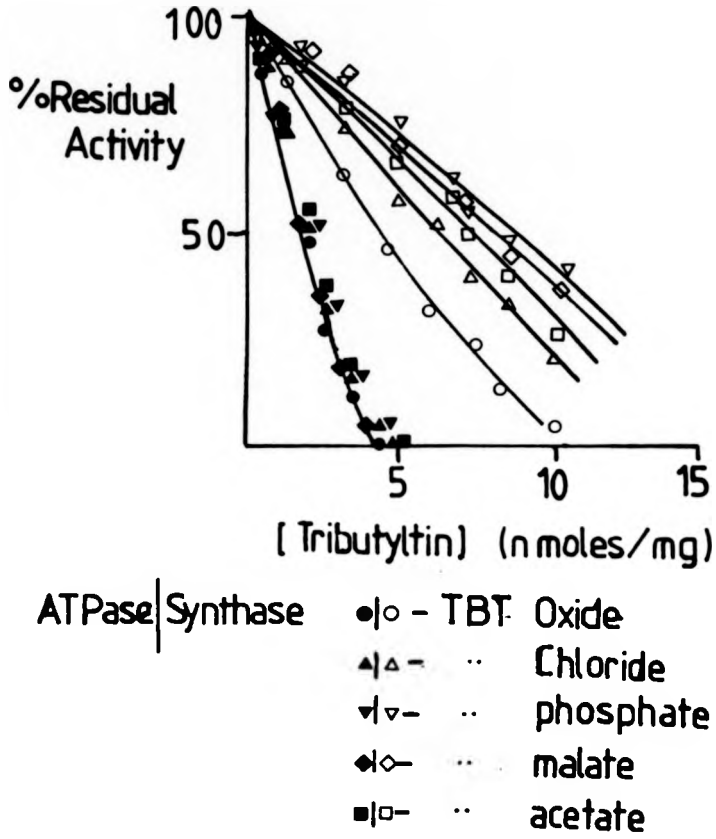
most potent compound in affecting mitochondrial ATP hydrolysis and synthesis, with I_{50} values of 1.0 and 2.5 nmoles/mg respectively.

It is apparent from the I_{50} values of the n-alkyltins that the peak of inhibitory activity is represented by the n-propyl and n-butyl derivatives, producing 50% maximal inhibition around 1.8-2.5 nmoles/mg and 7.6-8.0 nmoles/mg for ATP hydrolysis and synthesis respectively. Both shorter and longer alkyl groups are less active, although the triaryltin, triphenyltinchloride is equally potent. The peak potency of n-butyl and phenyl groups is also observed *in vivo* (Smith, 1982). By varying the anion of n-tributyltin, only slight differences are observed of 0.5 nmoles/mg, which are within the calculated deviation. The titration curves of these compounds are also similar and are presented in Figure 3i. In short, varying the anion of the trialkyltin appears to have a minimal effect on the 'oligomycin-like' activity of the compound.

Figure 3ii presents the titration curves for various n-trialkyltin chlorides which demonstrate their differential activities, as discussed above. Further, it is apparent that there is a differential effect on the ATP synthase and hydrolase functions of the mitochondrial ATPase complex. As reported earlier for beef heart submitochondrial particles, triorganotins cause differential inhibition of the ATP synthetic and hydrolytic activities of the $F_1 Fo$ ATPase (Emanuel *et al*, 1984). This effect can be clearly observed with DBCT in rat liver mitochondria, (titration curve presented in Figure 3iii), where specific concentrations of triorganotin will completely prevent ATP hydrolysis while allowing >80% of the control value of ATP synthesis, which is titratable by non-additive amounts of oligomycin, ossamycin and venturicidin (data presented in Table 3iv). These phenomena appear to be the properties of triorganotins in their direct inhibitory modes of action, since

Figure 3.i

Inhibition of ATP Synthesis and Hydrolysis in Mitochondria by n-tributyltins



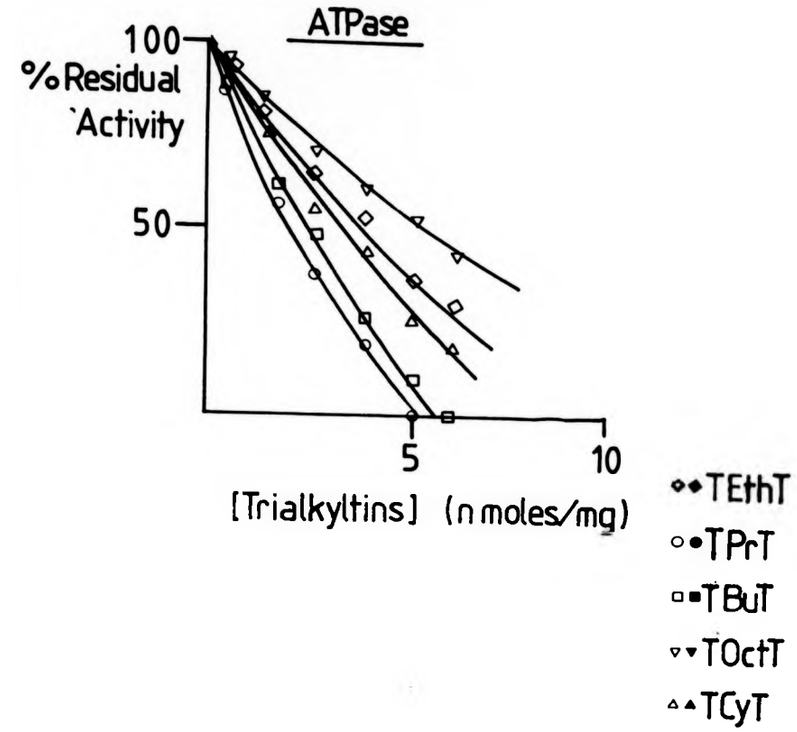
Legend:

These estimates were performed as described in 'materials and methods' and expressed as percentiles of control values (ATPase 33nmoles/mg/minute, ATP synthesis 88 nmoles/mg/minute).

Figure 3.ii

Inhibition of ATP synthesis and hydrolysis in Mitochondria by Various n-trialkyltins

Legend:
Estimates of ATP synthesis (hatched symbols) and ATP hydrolysis (open symbols) were performed as described in 'materials and methods' and expressed as percentiles of the individual control rates. ATP synthesis rates and ATP hydrolysis rates ranged between 75-101nmoles/mg/minute and 32-38nmoles/mg/minute respectively.



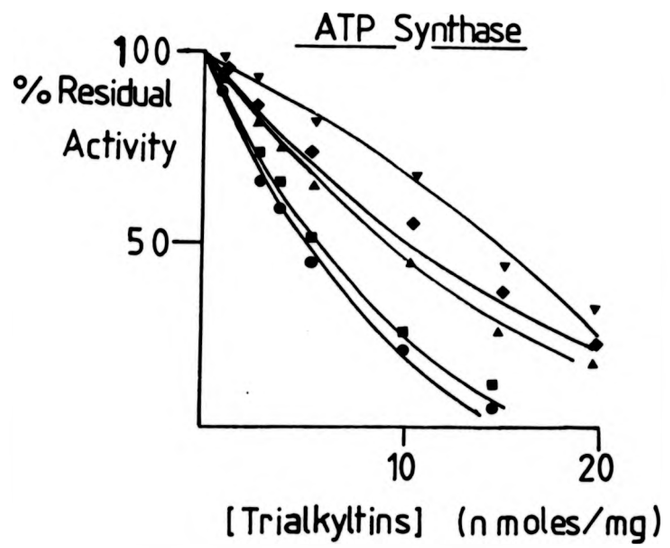
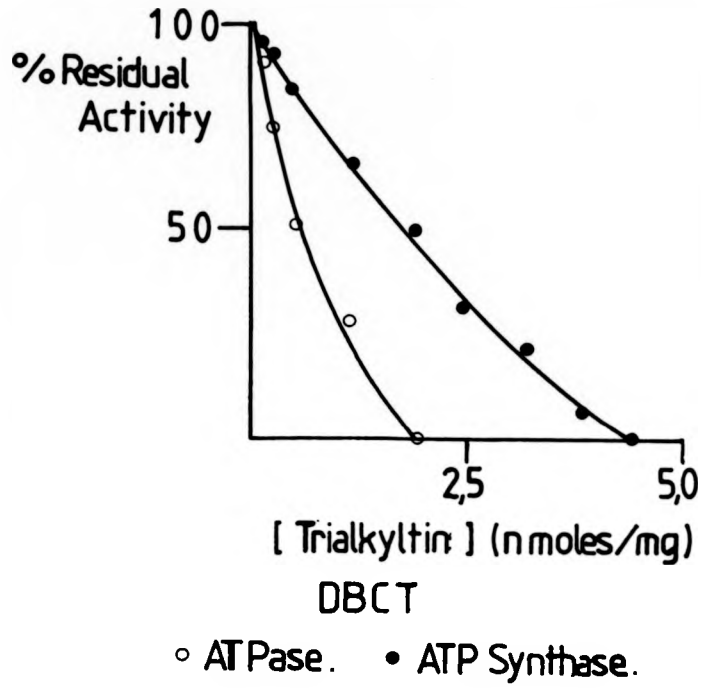


Figure 3.iii

Inhibition of ATP synthesis and hydrolysis in Mitochondria by DBCT



Legend:
Estimates of ATP synthesis (hatched circles) and hydrolysis (open circles) were performed as described in 'materials and methods' and expressed as percentiles of control rates, 38nmoles/mg/minute and 79nmoles/mg/minute respectively.

Table 3.iv

Differential Inhibition of ATP Synthesis and Hydrolysis by DBCT

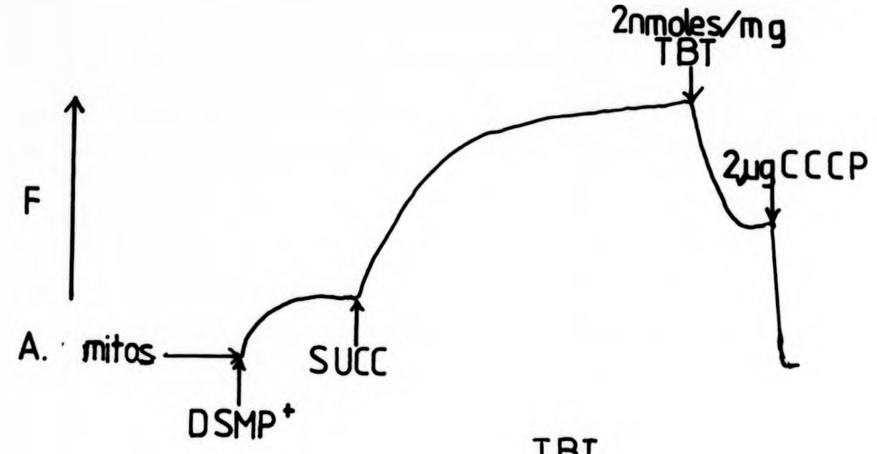
Inhibitors	ATPase (nmol/mg/min)	ATP synthase (nmol/mg/min)
None	35	84
DBCT	0	56
DBCT + oligomycin (2μgm/mg)	0	0
DBCT + ossamycin (4μgm/mg)	0	0
DBCT + venturicidin (2μgm/mg)	0	0

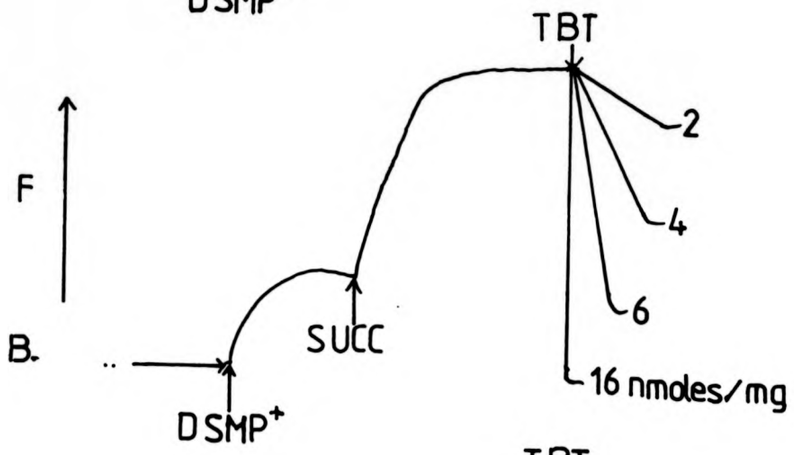
Legend:
Mitochondria were incubated on ice with 2.3 nmoles/mg DBCT before aliquoting into the ATPase and ATP synthase assays with or without antibiotic.

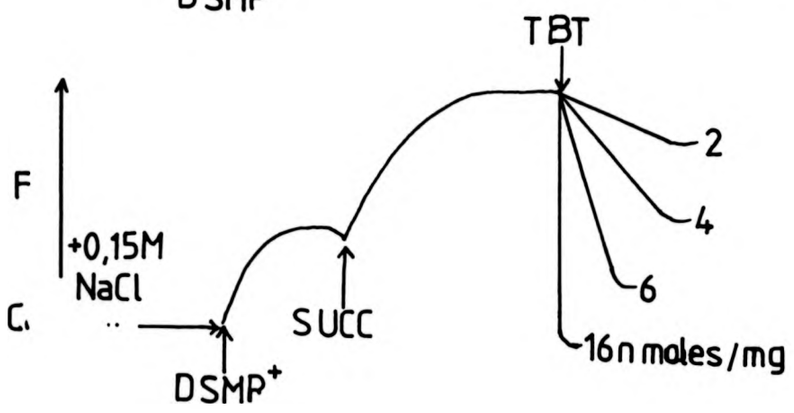
at these levels of triorganotin and halide, no competition is envisaged with non-specific or chloride/hydroxyl exchange mediated swelling. The less potent triorganotins do not appear to cause 100% ATPase inhibition, but allow 10% residual enzymatic activity while still maintaining control levels of ATP synthesis. The differential effect is still presumed present in absolute terms, since the enzymatic rates are still very different, 40:1 synthesis against hydrolysis in rat liver mitochondria. The above data questions the complete reversible nature of the mitochondrial F_1 Fo ATPase complex.

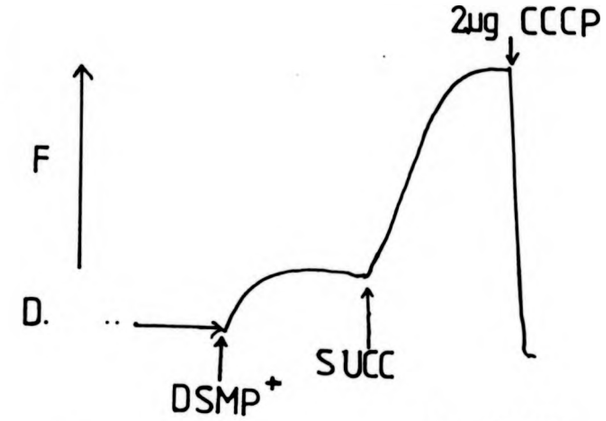
The Effects of Trialkyltins on Proton Motive Force

Considering the inhibitory functions of trialkyltins, these experiments were performed to rationalise their activities with the components of proton motive force (Δp), the driving force of ATP synthesis (Mitchell, 1961). Initial experiments were performed to monitor mitochondrial membrane potential ($\Delta\Psi$) with the fluorescent probe $DSMP^+$, as described in Chapter 2. Presented in Figure 3iv are a series of time dependent fluorescent traces demonstrating the action of tributyltinchloride. Figure 3iva demonstrates the step-wise depolarisation of membrane potential by tributyltin, the residual potential of which is sensitive to uncouplers, eg. CCCP. Figure 3ivb demonstrates that trialkyltins have the capacity to titrate mitochondrial membrane potential in an apparent concentration dependent manner. After the addition of limiting amounts of trialkyltin the membrane potential is depressed followed by equilibration at a lower level, the residual potential being sensitive to uncouplers or further additive amounts of trialkyltin. These effects were always additive independent of the triorganotin used, the net drop in potential is a function of the specific triorganotin concentration. The potency of particular triorganotins to lower mitochondrial membrane potential is assessed in Table 3v by their respective abilities









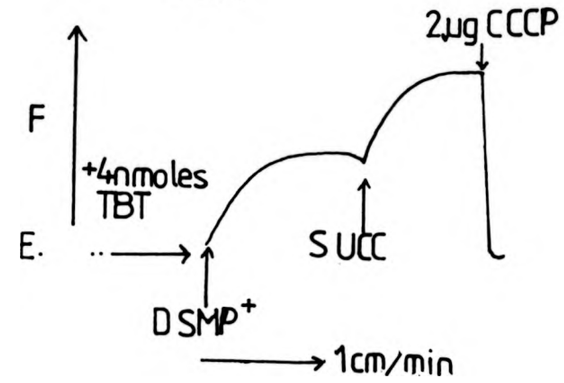


Figure 3.iv

The Effects of Trialkyltins on Mitochondrial Membrane Potential as Monitored by $DSMP^+$ Fluorescence

Legend:

Mitochondria were suspended at 1.0-1.25mg/ml protein in 250mM sucrose, 10mM HEPES pH 7.5, 8µM rotenone in 4ml four sided plastic cuvettes and placed in the fluorimeter (Ex 479 Em 589) before additions of 15 µl 0.2mM DSMP Br and succinate to make 5mM with rapid stirring. Inhibitor additions were made in ethanolic solution and never exceeded 10µl. In Figure 3.iv D mitochondria were preincubated with tributyltin on ice for 5 minutes before dilution into the assay.

to depress $\Delta\Psi$ by +100mV. These values are again expressed as the amount of triorganotins required per mg of mitochondrial protein. This ratio is again the criterion for triorganotin effect rather than net concentration.

These experiments were carried out in minimal halide conditions, therefore, the contribution of halide/hydroxyl exchange should be insignificant. When exogenous chloride is added to these assays the effect observed is negligible. Figure 3ivc undertakes the same titrations represented in Figure 3ivb in the presence of 0.15M NaCl, with little or no difference. Exogenous chloride (0.15M) did not appreciably alter the rate or steady state estimates of triorganotin mediated membrane potential depolarisation, performed using $DSMP^+$ fluorescence and the distribution of $[^3H]$ -$TPMP^+$ respectively. This result is not unexpected when one considers the mechanism of chloride imposed uncoupling via trialkyltins, chloride/hydroxyl exchange is essentially an electroneutral effect directed at the ΔpH component of proton motive force. However, it must be noted that Moore et al, 1980, have shown that high chloride medium does not effect transmembrane ΔpH shifts in plant mitochondria. Finally, Figure 3ivd demonstrates the precision of the trialkyltin titration, since preincubation with the inhibitor prior to $\Delta\Psi$ estimates predetermines $\Delta\Psi$ by producing a marked reduction in the energisation capacity of the membrane, the depression in the maximal obtained membrane potential corresponding to a similar titre of trialkyltin required to reduce $\Delta\Psi$ to an equivalent level.

As clearly seen in Table 3v, certain trialkyltins studied here have the capacity to cause $\Delta\Psi$ depolarisation at concentrations which do not exceed the 20 nmoles/mg required to perturbate the integrity of the membrane. The exceptions to this are trimethyltin, which remains largely ineffective, tripropyltin and triphenyltin. These compounds,

Table 3.v

The Potency of Various Triorganotins in Affecting $\Delta\Psi$

Triorganotin	+ 100mV $\Delta\Psi$ ± SD (nmoles/mg)
Trimethyltin chloride	300 ± 20.2
Triethyltin sulphate	19 ± 2.3
Tripropyltin chloride	30 ± 5.0
Tributyltin chloride	12 ± 2.2
Tricyclohexyltin hydroxide	11 ± 3.1

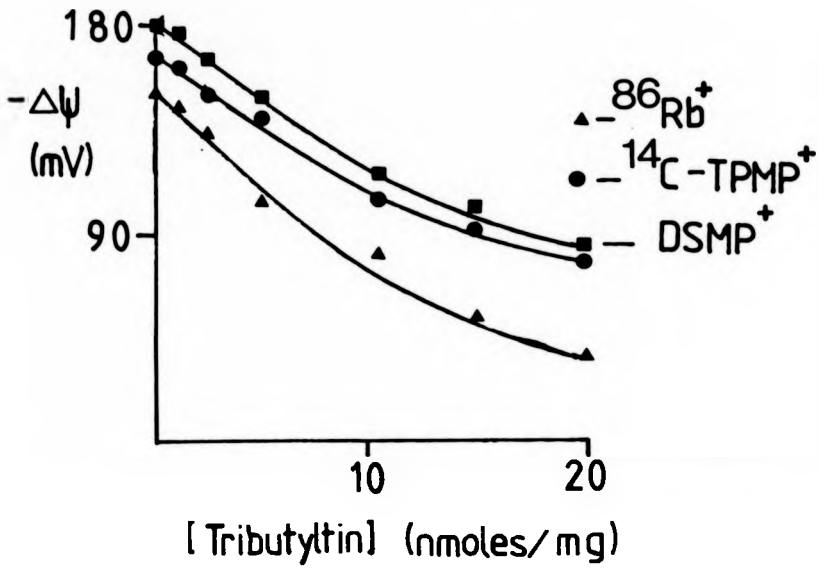
Legend:

The above assays were carried out as described in the legend to Figure 3.iv. Fluorescent changes were calibrated against $\Delta\Psi$ established using.the distribution of [^{3}H] -DSMP I, as described in Chapter 2. The concentrations represent the mean of 5 individual assays ± standard deviation.

although having potent effects on oxidative phosphorylation, do not cause $\Delta\Psi$ depolarisation as readily as other trialkyltin species. Considering the minimal action of chloride/hydroxyl exchange under these conditions, the ability of these compounds to bring about enzymatic inhibition, represents their capacity to directly inhibit the OS-ATPase complex in an 'oligomycin-like' fashion. However, trialkyltins which promote uncoupling, may have a dual mode of action, even when assayed under minimal chloride conditions. They may simultaneously inhibit the ATPase complex while causing profound uncoupling effects on mitochondria, which may effect estimations of their ability to produce enzymatic inhibition. The extremely active compound DBCT (4.5 nmoles/mg to produce +100mV reduction in $\Delta\Psi$), certainly will function as an uncoupler to bring about the cessation of oxidative phosphorylation, indeed, the concentration required to totally inhibit ATP synthesis (5 nmoles/mg) is very similar to that required to depolarise the membrane potential. Uncoupling may be the primary mode of action of DBCT on oxidative phosphorylation, however, it remains a potent inhibitor of the hydrolase reaction.

Further experiments were performed to measure steady state estimates of $\Delta\Psi$ and ΔpH utilising the distributions of $[^{14}C]$-lactate for ΔpH and of $[^{3}H]$-$TPMP^{+}$ and $^{86}Rb^{+}$ (in the presence of valinomycin) for $\Delta\Psi$ In general, the $\Delta\Psi$ estimates obtained from $DSMP^{+}$ fluorescence appeared higher than the latter two methods, particularly at lower membrane potentials. A typical set of titration curves with tributyltinchloride are presented in Figure 3v. The estimations of $\Delta\Psi$ differ by some 40mV between the lowest with $^{86}Rb^{+}$ and the highest with $DSMP^{+}$ for for a given titre of trialkyltin. A more pronounced version of this phenomenon has been commented upon previously by Mewes and Rafael, 1981, (cf. Chapter 2), observed when decreasing the mitochondrial

Figure 3.v



Variation in Δψ determination with $^{86}Rb^+$, [^{3}H]-TPMP$^+$ and DSMP$^+$ based methods on Titration with Tributyltin chloride

Legend:

Mitochondria were preincubated with tributyltin chloride and diluted into the assay. The respective assays were performed as described in 'materials and methods'. 2μM (1μCi) TPMP$^+$ (●) and 50μM (0.2μCi) $^{86}Rb^+$ (▲) were utilised in steady state estimates and 1μM DSMP$^+$ (■) used in time dependent fluorescence experiments.

membrane potential against a valinomycin mediated potassium diffusion potential. Certain differences in estimation may be inherent to each system rather than solely due to artefacts of high potassium concentrations, as suggested by Mewes and Rafael; not least the possible concentration of the inhibitor on centrifugation of the mitochondria with radioisotope based methods. Nevertheless, such deviations remain disturbing.

Estimation of $\Delta\Psi$, ΔpH and the resulting Δp are presented in Figure 3vi for titrations with various triorganotins. Two appropriate modes of action can be observed in Figure 3vi represented by DBCT and triphenyltinchloride. DBCT is observed to titrate Δp from the onset of inhibition, whereas triphenyltin has a more passive function until a relative concentration of 10 nmoles/mg, by which time ATP synthesis is only some 20% active. These observed differences are diagnostic of the two contrasting modes of action: triphenyltin acting as an 'oligomycin-like' inhibitor of oxidative phosphorylation; and DBCT operating principally by its uncoupling activity. The uncoupling function of triphenyltin is not maximised until 25 nmoles/mg are imposed upon the system, at which point the mitochondrial membrane may be disrupted and the enzymatic functions of the ATPase complex long since deceased.

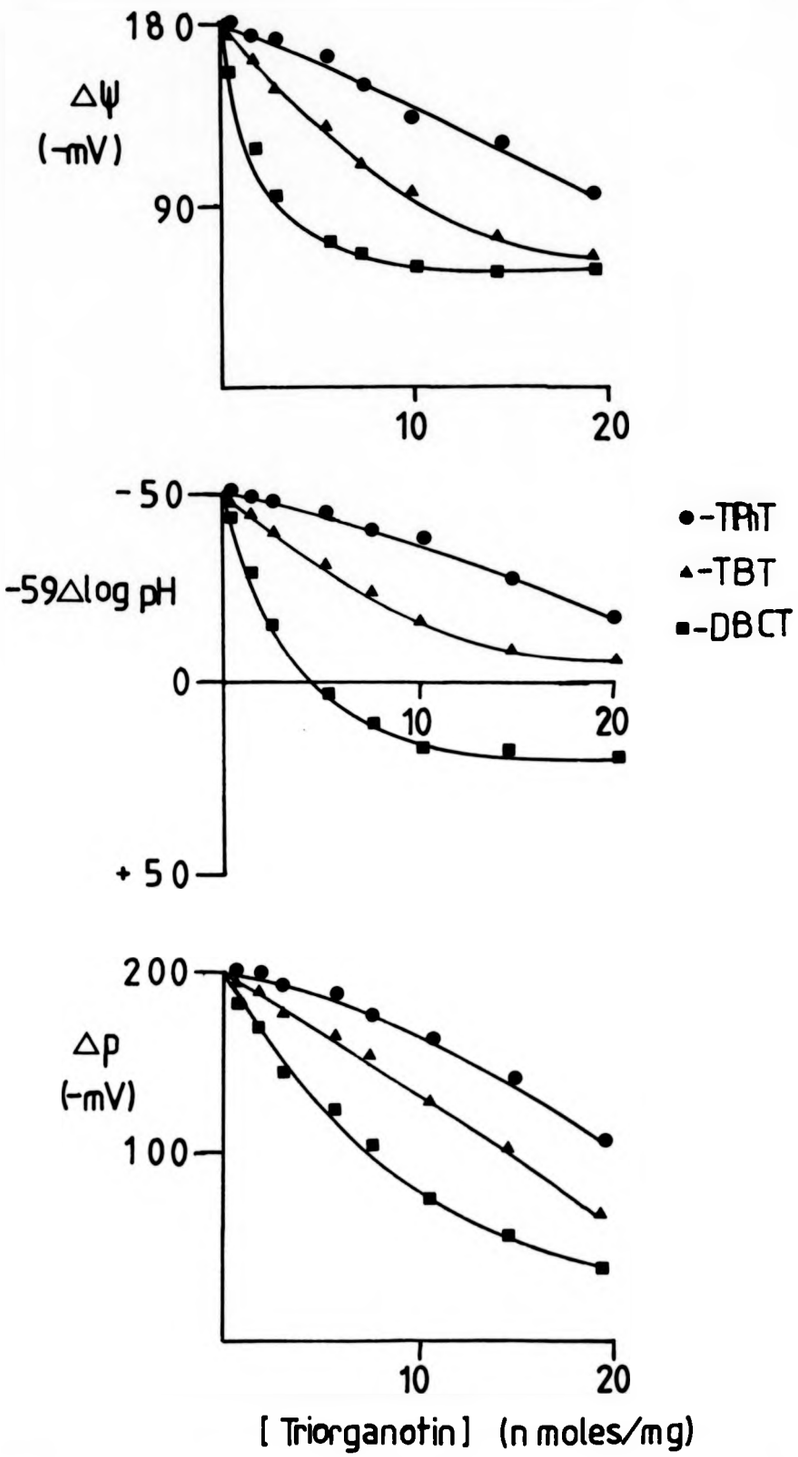
3.4 Discussion

It is clear from these results that the inhibitory mechanisms of triorganotins on mitochondria are complex, even in the absence of chloride/hydroxyl exchange. Triorganotins have been shown to inhibit proton conductivity in submitochondrial particles and chloroplasts (Papa et al, 1982; Gould, 1976 and 1978), presumably by their interaction with the Fo moieties of mitochondrial and chloroplast $^{+}$H-ATPase. These effects should occur at low titres of triorganotins, that is, those

Figure 3.vi

The Effect of Triorganotins on $\Delta\Psi$, ΔpH and Δp in minimal halide media

Legend:
$\Delta\Psi$ and Δp estimates were based on those obtained with [^{3}H]-TPMP^{+}. The distributions of 2µM (1µCi) [^{3}H]-TPMP^{+} and 25µM (0.5µCi) [^{14}C]-lactate were measured as described in 'materials and methods'.
(■) DBCT, (●) Triphenyltin chloride, (▲) Tributyltin chloride.



which are required to affect ATPase activity, since the present findings provide compelling evidence that at higher titres they will depolarise membrane potential, the very antithesis. These antagonistic effects are apparent between the individual activities of various triorganotins, as described earlier with the examples of DBCT and triphenyltin, which function at opposite ends of the spectrum. Therefore, the final activity of any triorganotin must be understood in terms of a composite mode of action. Nevertheless, it appears that most triorganotins will arrest proton conduction at the level of the H^+-ATPase at low titres, but at higher functional concentrations, will cause specific uncoupling and will ultimately destroy the integrity of the membrane, collapsing most energetic functions (the latter severe action being synonymous with the release of soluble protein and possible lysis (Wulf and Byrington, 1975)).

If a mechanism of direct trialkyltin mediated ion carriage is envisaged for uncoupling, a possible candidate for exchange is the tin-anion bond. The trialkyltin must be freely diffusible in the lipid bilayer and the putative exchange reaction should be pH and/or lipid phase dependent. Therefore, equilibria may exist which will ultimately re-equilibriate ionic imbalances across the membrane to facilitate a fall in membrane potential. The chemical nature of these interactions remain obscure at this time, particularly when one considers the anions oxide, chloride, acetate, phosphate and malate of tributyltin, all yield similar results. However, the observed effects may be mediated by a solvolysis product(s) of the original trialkyltin species which, especially in the presence of hydrophobic membranes, may yield a large number of complex molecular species (Garner et al, 1976; Hanson et al, 1980 and Alcock et al, 1985). Clearly, further chemical analyses are required to secure the exact nature of general organotin mediated

reactions within, or at the surface of hydrophobic phases.

Alternatively, the reduction in membrane potential may be a result of direct interaction of the trialkyltin species with an ion translocating component of the mitochondrial membrane, the function of which should remain independent of the external chloride concentration. Diwan, 1982 has reported that DBCT will enhance the rate of respiration dependent K^+ influx in rat liver mitochondria, a phenomenon which is pH dependent (Diwan and Tedeschi, 1975), but shows little dependence on chloride concentration (Diwan, 1982). Further studies by Diwan et al, 1983, have revealed DBCT to have a composite mode of action, titres of 4.5 nmoles/mg were required to affect respiration as determined by an oxygen electrode experiment, while higher concentrations were required to enhance radiolabelled $^{42}K^+$ influx. The value of 4.5 nmoles/mg required to inhibit respiration is remarkably similar to those required for the inhibition of ATP synthesis and uncoupling in the present study, the action of DBCT appears certainly multi-faceted. Nevertheless, at titres of DBCT > 5 nmoles/mg which apparently reduce $\Delta\Psi$ and promote unidirectional K^+ influx, the associated changes in charge distribution are at least consistent. It is also interesting to note that the effects of DBCT on K^+ influx were reversible by the monothiol 2-mercaptoethanol, which further preferentially reversed the K^+ influx activity over respiration. Further evidence that the site(s) of trialkyltin interaction may be at specific thiol residues. The site(s) of action have also been shown to overlap with the covalent inhibitor DCCD which produces an inhibitory effect on K^+ influx. This inhibitory activity is not expressed in the presence of DBCT (Diwan et al, 1983). The DBCT and DCCD binding sites on the ATP synthase complex have been shown to undergo some interaction (Kiehl and Hatefi, 1980; Partis et al, 1980), but the possibility remains that the factor(s)

responsible for K^+ transport may remain distinct. Jung et al, 1980 have shown that such sites are certainly distinct from the DCCD binding subunit of the ATPase complex (subunit 9) but this evidence however, does not rule out a complex interaction between several subunits of the ATP complex and/or other membrane components.

Aldridge et al, 1971 and 1977, have proposed that inhibition of the OS-ATPase is brought about by penta-coordinate binding of the trialkyltin compound, at an inhibitor binding site. Penta-coordination presumed to explain the relative ineffectiveness of tetraalkyltins in contrast to their tri- and dialkyltin analogues, on OS-ATPase. The presence of a fourth alkyl group sterically hinders penta-coordination and therefore, its binding to the inhibitor binding site. However, dialkyltins may undergo penta-coordination with a hydroxyl function, forming the fifth ligand and consequently, will bind to the inhibitory site. The effectiveness of each individual trialkyltin was also explained by the ease at which it may complex to the inhibitory binding site, where the optimal shape and sized alkyl group will be most effective. The optimal groups being represented by tri-n-propyl, n-butyl and phenyltin groups, which are good inhibitors of OS-ATPase activity, in contrast to trimethyltin which, although having the capability of penta-coordination, is a poor inhibitor. The lower binding affinity of trimethyltin against that of triethyltin in rat liver mitochondria (some ten times lower) was thought to represent this function (Aldridge and Street, 1970).

Although the trialkyltins studied here were found to be potent inhibitors of OS-ATPase and succinate driven ATP synthesis, the two enzymatic functions possess differential sensitivities. In all cases, the ATPase activity was found to be 3-4 times more sensitive to triorganotin inhibition than oxidative phosphorylation (Table 3iii). Further, the

results presented in Table 3iv for DBCT demonstrate that the residual ATP synthesis after the cessation of ATP hydrolysis, is sensitive to non-additive amounts of other established inhibitors of oxidative phosphorylation. These results are comparable to those reported by Emanuel et al, 1984, for beef heart submitochondrial particles. These results may be interpreted that the inhibition of the two processes are different, either chemically distinct, and/or spatially different. Separate sites for ATP synthesis and hydrolysis have been suggested by Penefsky, 1974; and Pederson, 1975 to exist on the mitochondrial ATPase complex. These suggestions were based on the observation that adenyl imidodiphosphate inhibited ATP hydrolysis but did not affect oxidative phosphorylation in submitochondrial particles. It is envisaged that the inhibitor will preferentially bind the site of ATP hydrolysis, causing the inhibition of OS-ATPase activity, but allowing continued ATP synthesis at another separate catalytic site (Pederson et al, 1978). However, a 'conformational model' may be evoked to explain the preceding results. In this model, ATP synthesis and hydrolysis occur at the same site, but the direction of the reaction is dependent on the conformation of the ATPase complex. The presence of trialkyltin may pertubate the reversibility of the two conformations, and preferentially maintain the ATP synthetic mode, at the expense of ATP hydrolysis; although the rates of each do not have to be comparable. While considering the above explanations, Emanuel et al, 1984, suggested that the residual ATP synthetic activity would be lost due to uncoupling. This may be true in the presence of chloride, but under the conditions maintained here, must depend upon the triorganotin. Indeed, DBCT should uncouple, but the functionally opposite triphenyltin should further inhibit the site of ATP synthesis. Further arguments regarding the inhibitory functions of triorganotins are presented on the penta-

coordinate complexes discussed in the following chapter.

3.5 Conclusion

The results presented in this chapter demonstrate that triorganotins are potent inhibitors of mitochondrial ATP hydrolysis and ATP synthesis. Triorganotins will also titrate membrane potential and the accompanying pH gradient in estimates of the proton motive force of isolated rat liver mitochondria in an apparent concentration dependent manner, in the absence of halide/hydroxyl exchange. Under these conditions the concentrations of triorganotin required to produce a drop of +80 to +120mV in Δp approach, or are in excess of, those required to inhibit ATP synthase activity, which are at least three fold greater than those which inhibit ATP hydrolase activity. The addition of exogenous chloride ion did not appreciably alter the steady state or rate estimates of triorganotin mediated $\Delta\Psi$ depolarisation. The evidence indicates that triorganotins possess an uncoupling effect which is independent of halide/hydroxyl exchange, and may not affect inhibition of the ATPase complex. The activities of various triorganotins may be best understood according to their abilities to uncouple or directly inhibit oxidative phosphorylation at the enzymatic level in the absence of halide/hydroxyl exchange.

CHAPTER 4

The Effects of Penta-coordinate Organotin Complexes on the Energetic Functions of Rat Liver Mitochondria

As discussed in Chapter 3, Aldridge et al, 1971 and 1977, have proposed that inhibition of the OS-ATPase complex may be brought about by penta-coordinate binding. Indirect evidence for the above proposal has come from binding studies of trialkyltins with rat and cat haemoglobins (Rose, 1969; Elliot et al, 1977 and 1979). Triethyltin binds to rat and cat haemoglobin with an affinity constant of 10^5-10^6 M^{-1} which is thought to be the result of interactions with histidine and thiol groups penta-coordinately (Rose, 1969 and Elliot et al, 1977), as well as two thiol groups, under certain conditions (Elliot et al, 1979). Penta-coordinacy is further supported by Mossbauer studies, and the lack of competition between triethyltin and penta-coordinate compounds (Elliot et al, 1979). Similarly, histidine residues are thought to be functional in the penta-coordinate binding of triethyltin to pyruvate kinase (Davidoff and Carr, 1973). However, Mossbauer studies on the ATPase inhibitory site of triethyltin on rat liver mitochondria suggested the interaction was four-coordinate. Internally coordinated triethyltin analogues exerted potent ATPase inhibition only as long as one coordination position was free, suggesting that only one bond is formed between triethyltin and its inhibitory site (Farrow and Dawson, 1978).

Aldridge further reported internally penta-coordinate triorganotin compounds as highly effective inhibitors of mitochondrial ATPase (Aldridge, 1978, and Aldridge et al, 1981), and suggested that because of their activity, binding must be four-coordinate. Similarly, trialkyltins may produce four-coordinate species.

The following chapter describes the activities of several internally penta-coordinate organotins and, in particular, the function and synthesis of (2-[(dimethylamino) methyl] phenyl) dialkyltin species, which have proven to be extremely potent inhibitors of the mitochondrial ATPase complex (Aldridge et al, 1981 and Emanuel et al, 1984).

Materials

All chemicals used were of analytical grade. The internally coordinated ester tins, mono-, di- and tri- (β-carbobutoxyethyl)-tin (BuAc) and (β-carbomethoxyethyl)-tin (MeAc) chlorides (Hutton and Oaks, 1976; Burley and Hutton, 1981), were gifts from Akzo Chemie UK Ltd. (2-[(dimethylamino)methyl] phenyl) dimethyl- diethyl- and dipropyl- tin halides (coded as the 'Ve' series), were synthesised in the laboratory using the dialkyltin dichlorides supplied by Ventron Corporation USA, n-butyl lithium and N'N'-dimethylbenzylamine were purchased from Aldrich, UK. All other special reagents and inhibitors were obtained as listed in Chapters 2 and 3.

Methods

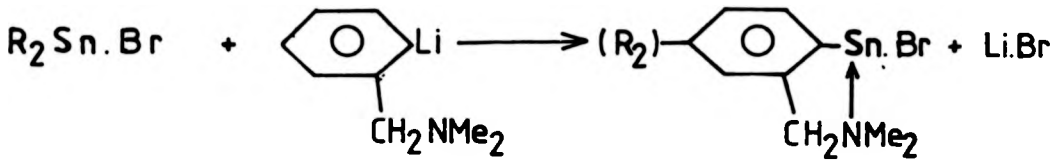
Enzymatic methods are described in Chapter 3. The methods used for estimating membrane potential and pH gradients are described in Chapters 2 and 3.

Synthesis of (2-[(dimethylamino) methyl]phenyl) dialkyltin halides (Ve series)

The syntheses were modified from those of Van Koten et al, 1975. 30 mmoles of butyllithium in 19ml of n-hexane were added to 75ml of dry ether under a nitrogen atmosphere to which was added 4.06g of N'N'-dimethylbenzylamine dropwise with stirring. This solution was stirred at room temperature overnight to produce a yellow viscous solution, which was in turn added dropwise to a solution of 30 mmoles

of dialkyltindihalide in 20ml ether at 0°C under nitrogen. The resulting colourless solution was stirred for three hours at room temperature. Colourless crystals of lithium bromide were formed and removed by filtration before evaporating the ether to yield a light yellow viscous oil which crystallised spontaneously. These crystals were further stirred with n-pentane before filtering and drying to produce a white product with a melting point of 105-106°C (yields between 60-65%).

Using the above protocol the methyl, ethyl, propyl bromides and ethyl chloride derivatives were synthesised. The dialkyltindibromides were produced from the dichloride species by exchange with sodium bromide in ethanolic solution, whereupon solid sodium chloride crystals were removed by filtration.



R = alkyl group

The products of these reactions were examined by elemental analysis (performed by Akzo Chemie at Duren) and high field 400MHz ^{13}C-and ^{1}H-NMR (Bruker WH400 located at Warwick) to confirm their structures and purity (99.50 to 99.90%). The ^{1}H-NMR of (2- [(dimethylamino)methyl] phenyl) diethyltin chloride (Ve2283(Cl)) is presented in Figure 4i, together

Figure 4.i

^{1}H-NMR and Proposed Structure of (2- (dimethylamino) methyl phenyl) diethyltin chloride

Legend:

A. 400MHz ^{1}H-NMR of Ve2283(Cl) 50mg in CD_3OD.

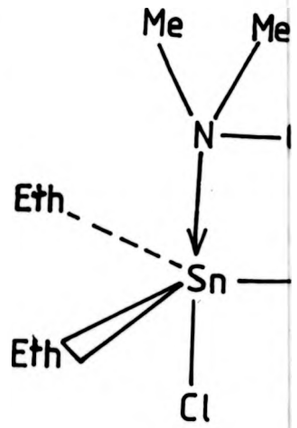
B. Proposed structure of Ve2283(Cl), in which the tin atom is five-coordinate as a result of intramolecular Sn-N bond formation, and in which the more electronegative ligands occupy axial positions (confirmed by Van Koten et al, 1980 utilising X-ray crystallography with Ve2283(Br)).

Figu

Figure 4.i

A.

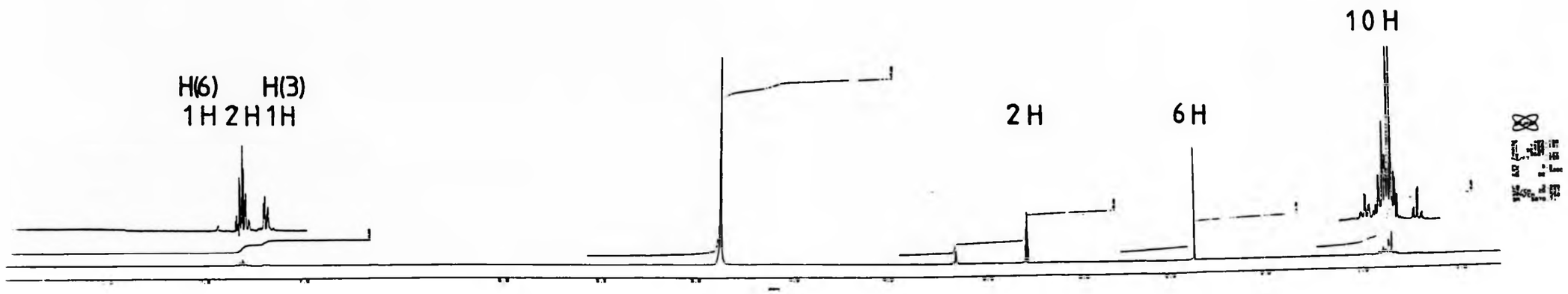
B.



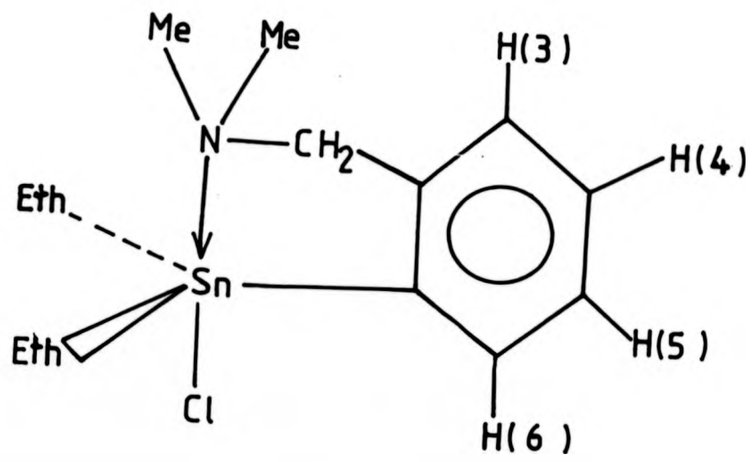
Ve2

Figure 4.i

A.



B.



Ve2283

with its assignments. The assignments of the R-Sn CH_2-N and CH_3-N resonances from integral values. However, previously unreported couplings of 27Hz and 56Hz were observed around the R-Sn resonances, presumably due to $^{119,\ 117}$Sn-C-C-H and Sn-C-H couplings respectively. The downfield resonances are assigned H(3), (a single proton at the highest aromatic region resonance 7.08 δ/ppm, ortho to the heteroatom), H(6), (a single proton at the lowest field resonance, 8.07 δ/ppm, para to the heteroatom), and H(4) H(5) which resonate as a multiplet between the latter (7.30 δ/ppm).

Preparation of Rat Liver Submitochondrial Particles

Rat liver mitochondria were diluted to approximately 10mg/ml mitochondrial protein in 250mM sucrose 10mM HEPES pH 7.5, before sonication for two minutes on ice (6 x 20 second bursts with cooling periods of 20 seconds) in an MSE 60W sonicator at maximum amplitude.

The resulting suspension was then recentrifuged at 12K r.p.m. in a Sorvall SS-34 rotor for 10 minutes at 4^{o}C. The supernatant was then centrifuged at 40K r.p.m. in a Beckman L5-65 centrifuge using the 42.1 Ti or 50 Ti rotors. The pellet from the above centrifugation was again resuspended in 250mM sucrose, 10mM HEPES pH 7.5 and recentrifuged as above. The resulting submitochondrial particles were resuspended as above, at approximately 25mg/ml for enzymatic analyses and storage at -80^{o}C, or solubilised directly with 1% Triton X-100, 10% CD_3OD in D_2O. Insoluble matter was removed by centrifugation at 40K r.p.m., as above.

Detection Ve2283 (Cl) binding to SMP using ^{119}Sn-NMR

The penta-coordinate tin complex Ve2283 was dissolved to saturation in CD_3OD (≈ 60mg/ml) and overnight ^{119}Sn-NMR spectrum obtained using the Bruker WH400 spectrometer in conjunction with a multi-nuclear probe. Similarly, Ve2283 was dissolved in 1% triton X-100, 10% CD_3OD

in D_2O with and without bovine serum albumin or submitochondrial protein (30mg/ml). Spectra of each of the samples were collected consecutively, around the chemical shift recorded for Ve2283 in deuteromethanol.

4.3 Results

The Inhibition of ATP Synthase and Hydrolase Activities

Table 4i demonstrates the inhibitory functions of the penta-coordinate tin complexes. Clearly, those compounds based on N'N'-dimethylbenzylamine nitrogen coordination are the most potent. The oxygen facilitated penta-coordinacy of the (β-carboalkoxyethyl)-tins adds little to the potency of these compounds. Comparisons of these activities with n-alkyltins demonstrates they are indeed less active inhibitors of ATP hydrolysis and synthesis in rat liver mitochondria. However, the penta-coordinate complexes with nitrogen coordination are significantly better inhibitors, Ve2283 (Br) has I_{50} values of 0.10 and 1.2 nmoles/mg mitochondrial protein for ATP hydrolysis and synthesis respectively, which makes it one of the most potent inhibitors of the ATPase complex known. Clearly, penta-coordinacy *per se* does not lead to an increased inhibitory activity towards the ATPase complex.

In Table 4i the differential sensitivity of the ATP synthetic reaction against that of the hydrolase reaction is observed. This phenomenon is clearly visible with the penta-coordinate complex Ve2283 where the concentration of organotin required to inhibit ATP synthesis (2.5 nmoles/mg) is 2½ fold that required to completely inhibit ATP hydrolysis (1.0 nmoles/mg protein). The conditions employed for these assays were performed in minimal chloride media similar to those discussed in Chapter 3 and should not facilitate chloride/hydroxyl exchange. The titration curve of Ve2283 (Br) is presented in Figure 4ii, together with that of the potent inhibitor DBCT for comparison.

Further comparisons of inhibitory actions of Ve2283 and DBCT are

Table 4.i

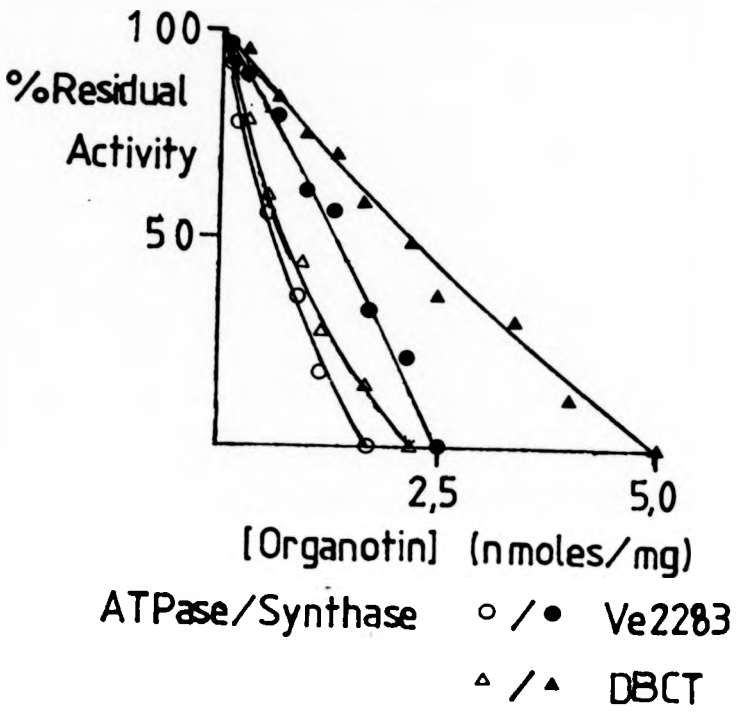
Legend:
ATPase and succinate driven ATP synthase activities were estimated as described in 'materials and methods' (Chapter 3). The numbers in parentheses correspond to the numbers of separate duplicate experiments. Control ATPase and ATP synthase rates ranged between 30-40nmoles/mg/minute and 80-100nmoles/mg/minute respectively. Values >50nmoles/mg are expressed at their solubility limits in aqueous solution.

The Sensitivities of Mit

Triorganotin

5-coordinate
'N'-coordinate
(2-[(dimethylamino) meth
- dimethyl
(Ve2283) - diethylt
(Ve2281) - diethylt
- dipropyl
'O'-coordinate
(β-carbobutoxyethyl)
Tri - tin chlo
Di - tin dich
Mono - tin tric
(β-carbomethoxyethyl)
Tri - tin chlo
Di - tin dich
Mono - tin tric
4-coordinate
Triethyltin chl
Tripropyltin ch
Tributyltin chl
DBCT
Triphenyltin ch

Figure 4.ii



Inhibition of Mitochondrial ATP Synthesis and Hydrolysis by Ve2283 and DBCT

Legend:
The enzymatic estimates were performed as described in 'materials and methods' of Chapter 3 (ATPase open symbols, ATP synthase hatched symbols) and expressed as percentiles of control values (ATPase 31nmoles/mg/minute, ATP synthesis 94nmoles/mg/minute).

presented in Table 4ii where their activities were monitored in rat liver submitochondrial particles. The I_{50} values for the ATP hydrolase are increased compared to those obtained with whole mitochondria, which may be indicative of an increased ATPase to protein ratio in SMP. However, when one considers the ATP synthetic activities, the I_{50} values for Ve2283 and DBCT do not increase proportionally; this may be indicative of an uncoupler mode of action on oxidative phosphorylation (certainly for DBCT, see Chapter 3), or the inhibitor binding site is in competition with non-specific binding prior to saturation. Total inhibition of ATPase activity by Ve2283 in rat liver submitochondrial particles occurs between 0.6 and 1.0 nmoles/mg protein. Estimates of around 0.5 nmoles/mg protein for the content of Fo in beef heart submitochondrial particles have been made (Slater, 1974), extrapolating these values to rat liver SMP (which are probably high), would suggest that Ve2283 is interacting with the ATPase complex between the ratios of 1:1 and 1:2, which makes the compound a valuable and sensitive probe for the mammalian F_1Fo-ATPase.

Figure 4iii demonstrates the titration of ATPase and ATP synthetic activities of submitochondrial particles by Ve2283 (Br). Over the range of concentrations presented here the ATP synthetic titration curve is tending towards a sigmoidal shape, that is, the compound produces a steep mid-point of titration with less pronounced effects at the extremes of high and low activity. This behaviour has been noted previously for the inhibitory behaviour of the antibiotic oligomycin (Ernster *et al*, 1963; Lardy *et al*, 1964 and Zoratti *et al*, 1982), but is by no means common to all inhibitors of the ATPase complex. The results are consistent with those of Aldridge *et al*, 1981, on rat liver mitochondria.

Table 4.ii

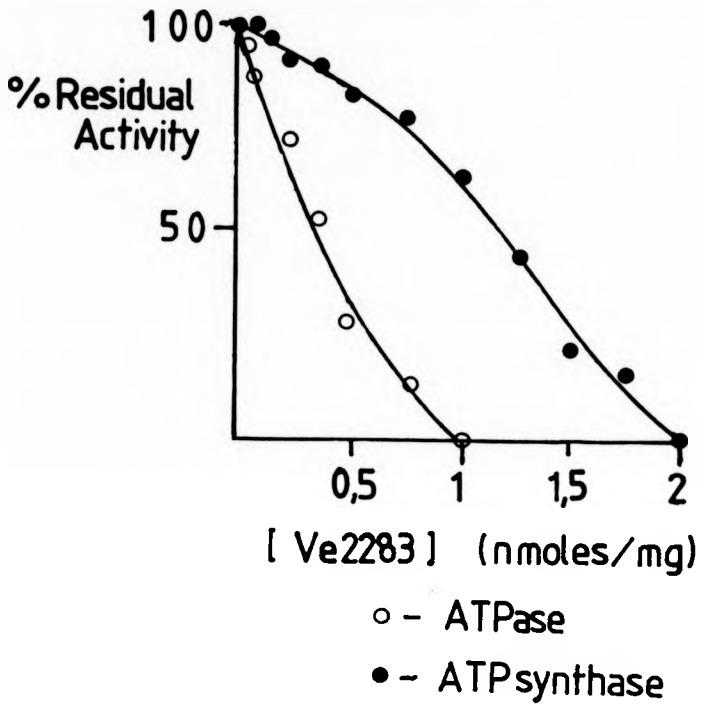
Inhibition of ATP Synthesis and Hydrolysis in Rat Liver Submitochondrial Particles

Triorganotin	pI_{50} (nmoles/mg protein) ± SD	
	ATPase	ATP synthesis
Dibutylchloromethyltin chloride	1.8 ± 0.18	2.5 ± 0.4
Ve2283 (Br)	0.2 ± 0.01	1.2 ± 0.2
Ve2283 (Cl)	0.2 ± 0.02	1.2 ± 0.2

Legend:
Submitochondrial particles were prepared as described in 'materials and methods' of this chapter. Each value presented is the mean of two separate duplicate experiments. ATPase and ATP synthase rates ranged between 56-58 nmoles/mg/minute, and 75-83 nmoles/mg/minute respectively.

Figure 4.iii

Titration of ATP synthetic and hydrolytic activities in Rat Liver Submitochondrial Particles by Ve2283 (Br)



Legend:
The enzymatic estimates were performed as described in Chapter 3 and the preparation of submitochondrial particles described in 'materials and methods'. The ATPase activity (open circles) was 58nmoles/mg/minute and the ATP synthase activity (hatched circles) was 83nmoles/mg/minute.

The Effects of Penta-coordination Complexes on Proton Motive Force

The methods of isotope distribution ($^{86}Rb^+$ [^{3}H]-TPMP$^+$ and [^{14}C] lactate; cf. Chapter 2) and time dependent DSMP$^+$ fluorescence have been applied. Table 4iii presents the organotin concentrations required to reduce the membrane potential by + 100mV.

In all cases, the penta-coordinate tins are poor uncouplers. Although it must be noted the ester tins are comparatively better at reducing membrane potential, which is possibly their mode of action on oxidative phosphorylation. In contrast to their potent inhibition of ATPase and ATP synthase the N-coordinate tins do not disturb the membrane potential until quite high concentrations (30 nmoles/mg are required to reduce $\Delta\Psi$ by + 100mV for Ve2283 (Br)). As argued in Chapter 3, such high inhibitor/protein ratios may seriously perturbate the integrity of the membrane, indeed, Ve2283 may not uncouple per se. The function of these inhibitors is thus analogous to that described for triphenyltin-chloride, in that they are direct inhibitors of the ATPase complex, only more potent. The nature of the tin-halide bond again appears not to affect the compound's inhibitory mode of action, Ve2283 (Cl) behaves similarly to Ve2283 (Br).

Table 4iii also displays titration variations imposed by different techniques of measurement, as noted earlier.

Comparing the functions of Ve2283 and DBCT, the 'mechanistic' differences of each become apparent. Both compounds inhibit ATP hydrolysis, the mechanisms of which we have no reason to believe are dissimilar, but their modes of action in halting oxidative phosphorylation certainly are (see Figure 4iv). DBCT is observed to titrate the membrane potential component of Δp at relatively low inhibitory concentrations (3.0-5.0 nmoles/mg), causing a decline in $\Delta\Psi$ from the onset of inhibition of oxidative phosphorylation. This is in direct contrast to Ve2283 which

Table 4.iii

The Abilities of Organotins to Reduce Membrane Potential

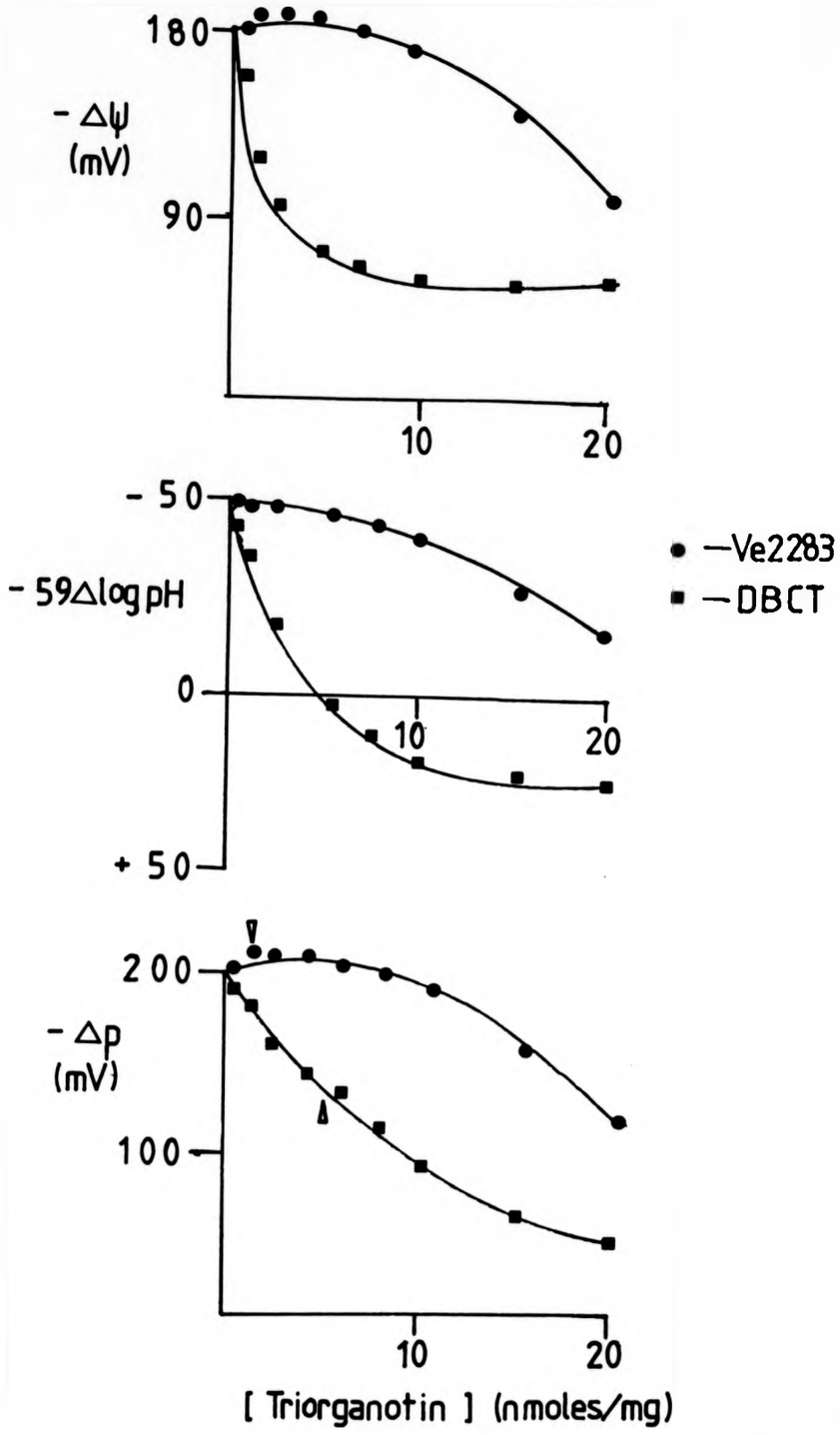
Triorganotin		+ 100mV $\Delta\Psi$ ± SD		
(2- [(dimethylamino) methyl] phenyl)				
	- dimethyltin bromide	20 ± 1.6	26 ± 1.4	30 ± 4.1
	- diethyltin chloride	26 ± 1.8	28 ± 1.3	30 ± 4.1
	- diethyltin bromide	26 ± 1.6	28 ± 1.3	30 ± 4.1
(β-carbobutoxyethyl)				
Tri	- tin chloride	25 ± 4.0	30 ± 3.5	30 ± 4.1
Di	- tin dichloride	-	-	>50
Mono	- tin trichloride	-	-	>50
(β-carbomethoxyethyl)				
Tri	- tin chloride	35 ± 4.1	45 ± 4.0	45 ± 4.1
Di	- tindichloride	-	-	>50
Mono	- tin trichloride	-	-	>50

Legend:
These assays were performed as described in 'materials and methods'.of Chapter 2. The fluorescence assay using $DSMP^+$ was undertaken 4 times and the average value presented. The values for the ^{86}Rb and $[^3H]$-$TPMP^+$ experiments are the mean of three separate duplicates. Values >50nmoles/mg are expressed at their solubility limits in aqueous solution.

Figure 4.iv

The Effects of DBCT and Ve2283 on $\Delta\Psi$, ΔpH and Δp

Legend:
$\Delta\Psi$ and Δp estimates were based on those obtained with [^{3}H]-TPMP$^+$. The distributions of 2 µM [^{3}H]-TPMP$^+$ (1 µCi) and 25 µM [^{14}C]-lactate (0.5 µCi) were measured as described in Chapter 3 (●, Ve2283 (Br); ■, DBCT). The arrows represent the values at which ATP synthesis is completely inhibited.



actually produces a slight nett increase in Δp (-15 to -20mV as ascertained by [^{3}H]-TPMP and [^{14}C]-lactate distributions), before causing a progressive reduction. This type of behaviour is consistent with Ve2283 having a direct interaction on the proton channel of the mitochondrial ATPase complex and DBCT as an active uncoupling agent. The high inhibitor concentrations which are necessary for Ve2283 to cause membrane potential depolarisation are at least one order of magnitude larger than those required for enzymatic inhibition, which indeed, as argued in the previous chapter, may result in the loss of the integrity of the membrane, rather than a bona fide uncoupling action.

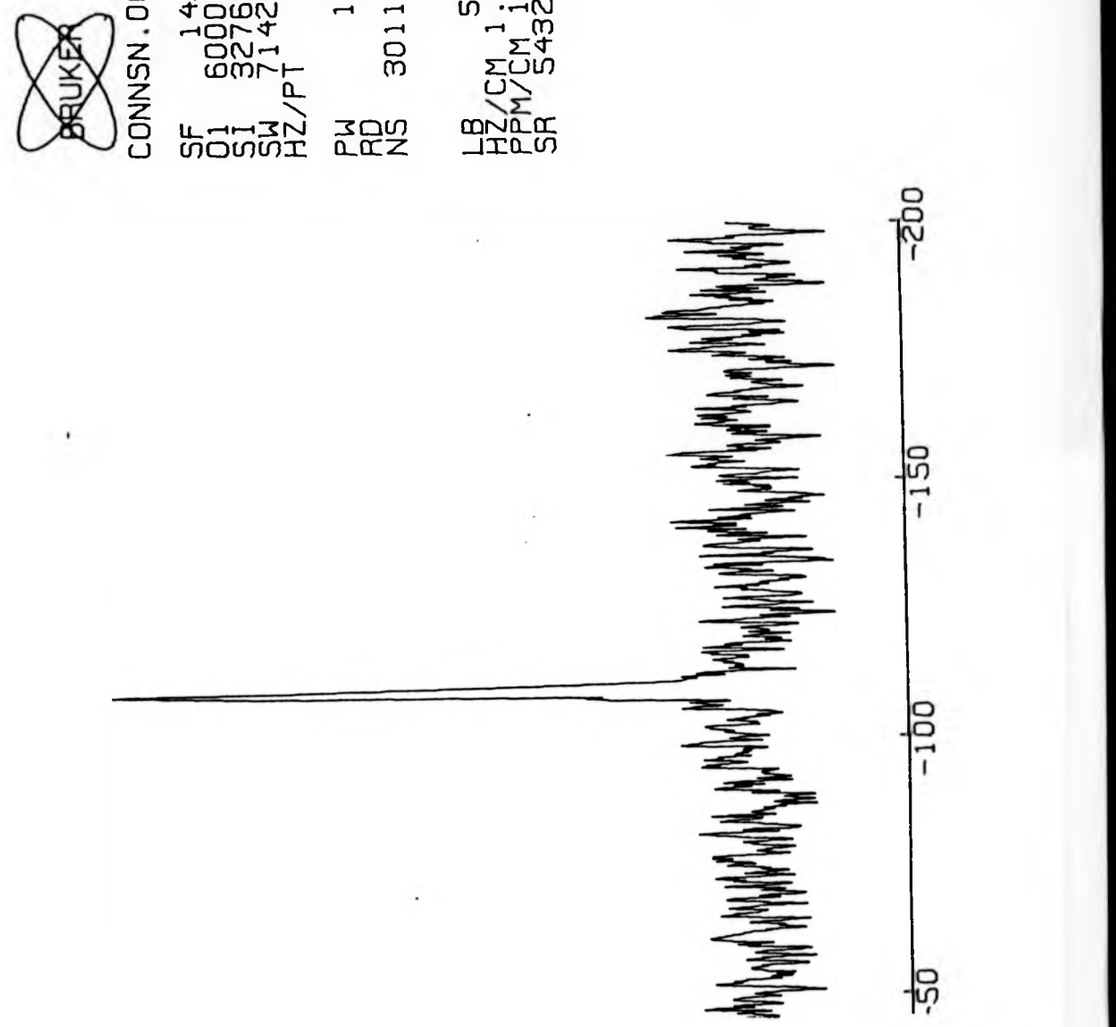
The Detection of Penta-coordinate Tin Binding

Figure 4v presents the ^{119}Sn NMR of Ve2283 (Cl) in duteromethanol. The spectrum consists of a single decoupled peak at -109.48 δ/ppm. This chemical shift was then used as a standard for the following two experimental spectra obtained for Ve2283 (Cl) in duterated water with 10% CD_3OD and 1% triton. These conditions were used to optimise the differing solubilites of the organotin complex and soluble protein extracted from successive preparations of rat liver submitochondrial particles. The chemical shift recorded in duteromethanol was consistent for an internally coordinated organotin complex (-90 to -110 δ/ppm, unpublished data from the International Tin Research Institute).

Figure 4vi A and B presents the spectra for Ve2283 (Cl) in the presence of 10% CD_3OD, 1% triton in D_2O plus and minus soluble protein from rat liver submitochondrial particles respectively ($\approx$ 50mg). The effect of changing the solvent conditions from CD_3OD was to move the chemical shift by approximately 2.5 δ/ppm to -106.96 δ/ppm, the addition of protein made only a slight further difference, -106.45 δ/ppm. Despite little change in the chemical shift a change in the spectrum in the presence of protein was noted. The generation of satellites around

Figure 4.v

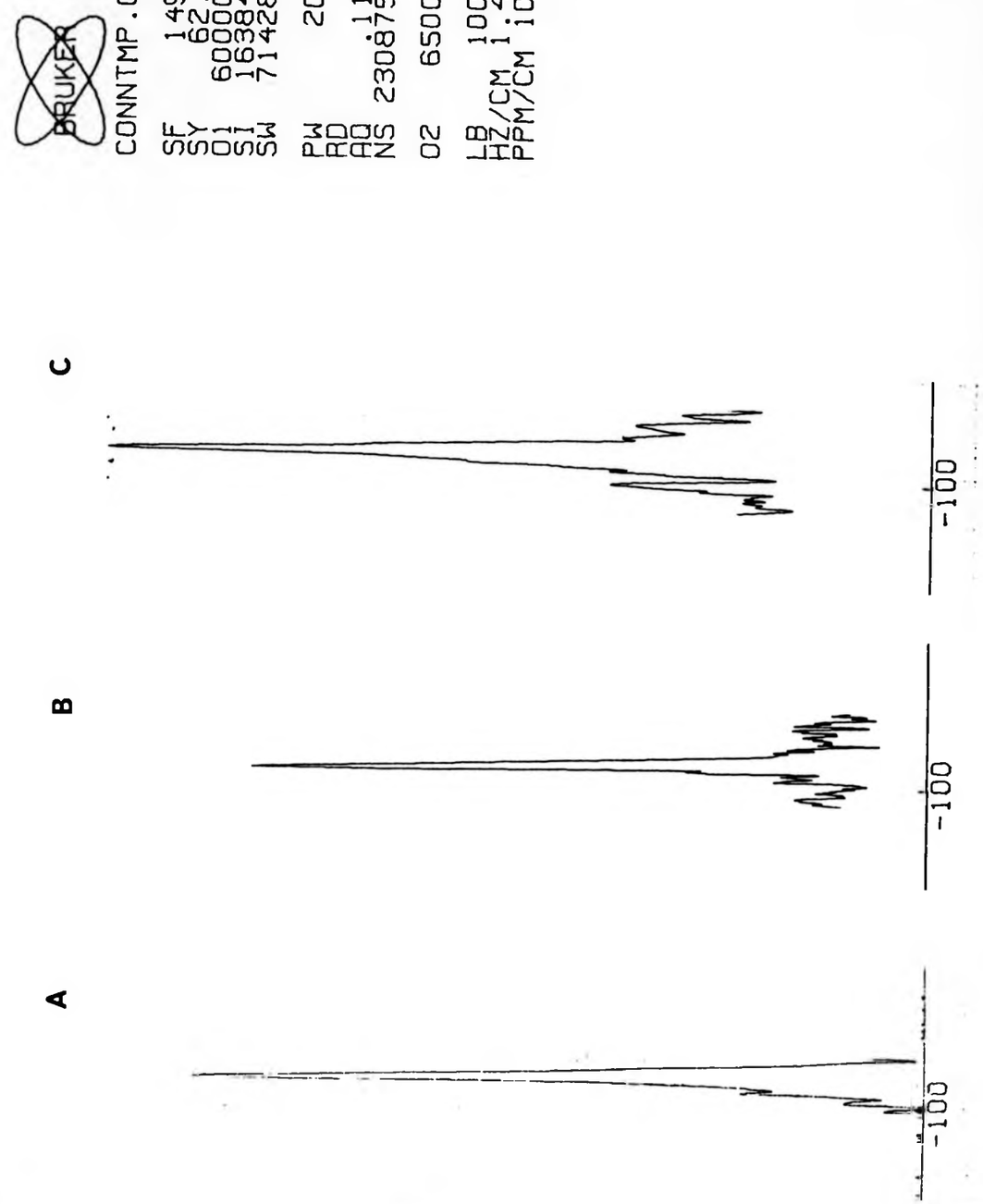
^{119}Sn-NMR Spectrum of Ve2283 (Cl)



Legend:
400MHz ^{119}Sn-NMR of Ve2283 (Cl), 50mg in CD_3OD

Figure 4.vi

^{119}Sn-NMR Spectra of Ve2283 (Cl), the Effects of Soluble Mitochondrial Protein



Legend:

A. 400MHz ^{119}Sn-NMR of Ve2283 (Cl), 50mg with 10% CD_3OD, 1% Triton in D_2O; total volume 2ml.

B. As described in A. with the addition of 50mg of bovine serum albumin.

C. As described in A. with the addition of 50mg of soluble mitochondrial protein.

the central ^{119}Sn peak in spectrum 'B' (J 1.043 and 1.788Hz) would suggest that there are two separate tin based molecular species (the new species would have to be of insufficient quantity to generate a further peak, or of similar chemical shift) or perhaps the same species in two very different environments. Although speculative, both the above interpretations are consistent with the penta-coordinate tin interacting with a mitochondrial membrane component. The component should be specific, triton soluble and present in a reasonable quantity; conditions which do not preclude the ATPase complex.

Unfortunately, the solubilities of the components of this system means that we are approaching the limits of information which the above experimental approach may yield. Nevertheless, the above experiment has indicated that Ve2283 may interact chemically with the mitochondrial membrane, not just by virtue of its lipid solubility.

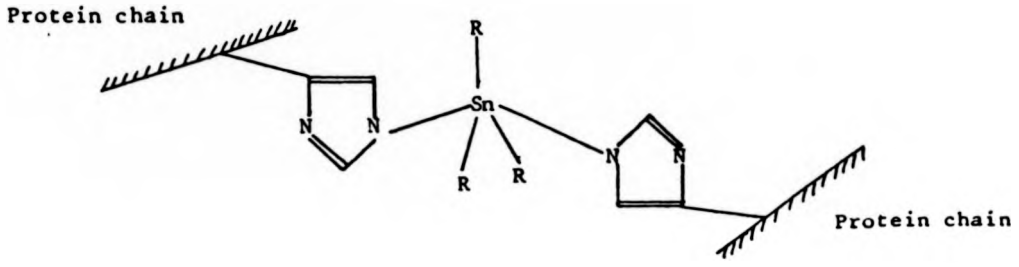
4.4 Discussion

The internally coordinated ester tins (o-coordinate) are relatively poor inhibitors of the ATPase complex. Nevertheless, they do inhibit in the relative order of tri-, di- and mono- β-corboalkoxyethyltin, which, because of their potential for penta-coordinacy could facilitate 4, 3 and 2 valent interactions with membrane components. Clearly, the 4-coordinate interaction would be favoured in terms of activity, the tri β-carboalkoxyethyltins are 3-4 fold more active inhibitors compared with the Di- β-carboalkoxyethyltin analogues.

Aldridge and Street, 1970, have postulated that the mechanism of tri-alkyltin binding to the inhibitor site on the OS-ATPase complex involves the formation of tin-nitrogen linkages between the trialkyltin and paired histidine residues (Figure 4.vii), similar to that proposed by Rose, (1969), for the binding of triethyltin to rat haemoglobin, and by Davidoff and Carr, (1973), to pyruvate kinase. However, Gould,

Figure 4.vii

Proposed Sn-coordination with Histidine Residues Within Proteins



Legend:
Proposed structure for trialkyltin binding to haemoglobin, pyruvate kinase and OS-ATPase.

(1978), has demonstrated that triphenyltin inhibition of OS-ATPase activity cannot be reversed by large excesses of exogenous histidine, and Siebenlist and Taketa, (1983), have concluded that triethyltin bromide ligands to neither cystein or histidine. Nonetheless, the possibility of polymeric coordination of histidine residues with trialkyltins should exist, since other nitrogen based heterocycles have been reported to undergo such reactions, (Paller, 1965 and Luijten et al, 1962).

Emanuel et al, (1984), have shown that DBCT inhibition of oxidative phosphorylation, OS-ATPase and ATP-driven transhydrogenase can be effectively reversed by dithiols and not monothiols in beef heart submitochondrial particles. Similar findings have been obtained by Cain et al, (1977), for dibutyltin dichloride. Gould, (1976), has also shown that triphenyltin inhibition of photophosphorylation and on decreased proton permeability in CF_1 depleted chloroplasts were specifically reversed by dithiols. Recently, Yagi and Hatefi, (1984), have reported the reversal of triphenyltin inhibitors with not only dithiols, but also β-mercaptoethanol. Although such results are not conclusive evidence of the role of vicinal dithiols function in triorganotin mediated inhibition of OS-ATPase, they are certainly suggestive of the interpretation. However, Nbf-Cl inhibition is also reversed by dithiothreitol, but is known to be a specific inhibitor of F_1-ATPase, modifying an essential tyrosine residue of the β subunit (Ferguson et al, 1974 and 1975). It must also be noted that the penta-coordinate complex Ve2283 does not react with dithiols or monothiols, as ascertained by the 2, 6-dichlorophenolindophenol technique of Aldridge and Cremer, 1955, (Carver, 1980, unpublished results), neither is the inhibition reversed by thiol compounds (Emanuel, 1981). The interaction therefore noted in results may be novel for triorganotins.

As outlined in 'Results', the N-coordinate compounds such as Ve2283 are exceptional in their capacity to inhibit the mitochondrial ATPase complex, without uncoupling. Indeed, when Ve2283 is introduced into the system Δp is seen to rise by -15 to -20mV at titres up to 4-5 nmoles/mg protein. This behaviour is in total contrast to that of DBCT which functions as an uncoupling agent, although both inhibit ATPase activity. Ve2283 is therefore preventing proton conduction, and as a consequence, is preventing the catalytic functions of the OS-ATPase complex. Other triorganotins may produce similar effects to lesser degrees but will be superceded as other uncoupling functions mitigate. Nevertheless, it appears that triorganotins will arrest proton conduction at the level of the H^+ ATPase at low titres, whereas at higher titres will cause uncoupling, and ultimately destroy the integrity of the membrane, collapsing most energetic functions (the latter severe action being synonymous with the release of soluble proteins and possible lysis (Wulf and Byrington, 1975)).

The phenomenon of continued ATP synthesis in the absence of the hydrolysis reaction has been addressed by Ferguson and Parsonage, (1984), for an inhibitor of the ATPase complex. They have postulated that an increase in Δp potentiated by the inhibition of protonic conductance through Fo may cause the inhibited ATP synthase enzymes to increase their turnover rate, and result in a compensated rate of oxidative phosphorylation over ATP hydrolysis. This explanation is possible when one considers the nett stimulation of Δp at low titres of Ve2283. However, the rate of ATP synthesis by the uninhibited enzymes would have to be truly drastic, particularly when one considers the situation in submitochondrial particles where 90% of the ATP hydrolytic capacity has been lost while oxidative phosphorylation is continuing at $> 80\%$ of the control rate. It must also be noted that unlike beef heart

mitochondria where ATPase rates can be as much as 25 fold the ATP synthetic rate, rat liver mitochondria produce rates of the same order of magnitude, thus comparisons of the ATP synthetic and hydrolytic functions may be considered directly without preferential turnover rates of the enzyme complex. Further, the depression of ATP hydrolysis by the back pressure of protonic conductance leading to ATP synthesis, i.e. the measurement of ATPase rates in the presence of Δp for mitochondria, does not unduly affect the inhibitor titration as evidenced by similar observations in submitochondrial particles (Figure 4iii and Emanuel et al, 1984). However, the prediction of Ferguson and Parsonage, (1984), may have some bearing on the phenomenon of increased rates of oxidative phosphorylation (10-20%) of submitochondrial particles in the presence of low titres of triorganotin (Emanuel, 1981).

The data on the differential inhibition of ATP synthesis and ATP hydrolysis can be interpreted in terms of dual pathways for protonic conductance through the Fo portion of the mitochondrial ATPase complex. Protonic conductance should occur by two chemically distinct pathways, distinguishable by their differential affinities for triorganotins. One path leading to the hydrolysis of ATP and possibly functioning in synthesis, and a second which is utilised solely for synthesis. Whether these entities are functionally mediated through conformational change in the complex, or via distinct protonic channels can only be speculated upon at the present time. Arguments continue as to whether protons interact with the bulk aqueous phase and are transported by specific ion channels, or move via localised pathways whereupon an interaction with the ATPase complex is envisaged. Within the chemiosmotic framework the above results should lead to the conclusion that two independent structural ion channels should be present on the ATPase complex, or that two functionally different complexes are present.

At the present time, of the ubiquitous eukaryotic proteins found in Fo, subunits 6, 8 and 9, two have had their capacities to conduct protons in a voltage dependent manner demonstrated in reconstituted lipid vesicles; J. Velours (personal communication), and Shindler and Nelson, (1982), for subunits 8 and 9 of Saccharomyces cerevisiae respectively.

Protein sequences and protein sequences derived from nucleic acid sequence data of Fo subunits, now exist for a variety of eukaryotic organisms. When these are analysed for their histidine and cystein contents, aminoacid residues which have been linked with the binding of triorganotins, these aminoacids appear sparingly. For example, in the yeast Saccharomyces cerevisiae, subunit 6 has four histidines and one cystein, subunit 8 has neither histidine nor cystein, and subunit 9 has one cystein and no histidine residues (data derived from DNA sequence of the mitochondrial genome, see Chapter 7 of this thesis). On this basis that interactions are possible with subunits 6 and 9 whereas histidine interactions are only possible with subunit 6. When various subunit 6 sequences are surveyed they display conserved histidine elements towards their carboxyl ends (cf. Table 4iv). This sort of interaction is attractive since the carboxyl side of subunit 6 may be of some functional import, based on mutational analysis of drug resistant mutants (see Chapter 7 of this thesis). If basic residue interactions are to be considered, for example the hydroxyl groups of serine residues, then all the above proteins have several such residues.

Of the triorganotins examined so far, Ve2283 is the most interesting, its specific inhibition of the OS-ATPase makes the compound of great value; particularly when one considers the lack of coincidental functions such as direct uncoupling, and chloride/hydroxyl exchange (Aldridge

Table 4.iv

Conserved Histidine Based Motifs in Subunit 6

Organism	NH_2	Motifs		COOH	
Human	LLPHSFT	LAHFLP	TAGHLL MHLIGS	LYLHDNT	Anderson et al, 1981
Bovine	LLPHSFT	LAHFLP	TAGHLL IHLIGG	LYLHDNY	Anderson et al, 1082
Rat	LLPHTFT	LAHFLP	TAGHLL MHLIGG	LYLHDNT	Grosskopf and Feldman, 1981
Mouse	LLPHTFT	LAHFLP	TAGHLL MHLIGG	LYLHDNT	Bibb et al, 1981
D.melanogaster		FAHLVP	IAGHLL		de Bruijin, 1983
D.Yakuba		FAHLVP	IAGHLL		Clary and Wolstenholme, 1983
S.cerevisiae		SAHLVF	LAGHLL	LYLH	This Laboratory

et al, 1981), at titres which bring about inhibition. Ve2283 radioactively labelled would make a useful affinity label for the H^+-ATPase complex. Attempts to achieve this goal by standard chemical isotope exchange on the ring protons of N'N'-dimethylbenzylamine (catalysed by borontrifluoride or aluminium chloride) have met with only limited success, some 12.5 to 18.5% as model duteration (ascertained by observing m+1 totals on mass spectrometry). A much higher incorporation is required for the analogue to be a good probe of the ATPase complex. Tritiation in steam appears a better alternative, although impractical under normal laboratory conditions.

4.5 Conclusion

(2-[(dimethylamino) methyl] phenyl) dialkylhalide derivations are good inhibitors of the mitochondrial ATPase complex in both its synthetic and hydrolytic capacities. Penta-coordination per se does not lead to good inhibitory qualities, but does lend evidence to the 4-coordinate binding of triorganotins to the ATPase complex, as evidenced by the ß-carboalkoxyethyltin halides. The N-coordinated complex Ve2283 does not effect a reduction in Δp until high titres of the inhibitor are present, at which time all enzymatic functions of the ATPase have deceased. The compound is a specific functional inhibitor of the ^+H-ATPase titrating the complex at approximately 1:1 or 2:1, suggesting a highly specific interaction or covalent binding. ^{119}Sn-NMR provides evidence that Ve2283 binds to a triton soluble component of the mitochondrial membrane. The N-coordinate tins also differentially titrate ATP hydrolysis and ATP synthesis in both rat liver mitochondria and submitochondrial particles, which is indicative of dually functioning H^+ATPase complex.

CHAPTER 5

The Effects of Trialkylleads on the Energetic Functions of Rat Liver Mitochondria

5.1 Introduction

As described for trialkyltins, the trialkylleads act on mitochondria by three basic mechanisms: namely, direct inhibition of the mitochondrial ATPase complex (Aldridge et al, 1977), stimulation of chloride/hydroxyl exchange (Coleman and Palmer, 1971, and Rose and Aldridge, 1972), and finally, by gross swelling of the mitochondrial membrane (Aldridge et al, 1977). Similarly to trialkyltins, the trialkylleads also show a peak of activity against the ATPase complex at tributyl and triphenyl on increasing the alkyl chain length (Aldridge et al, 1977). Overall, the trialkylleads appear to favour chloride/hydroxyl exchange in their mode of action, as ascertained by comparing relative concentrations at 50% maximal change (Aldridge et al, 1977), a possible exception to this is the triaryllead, triphenyllead chloride.

Although as stated above, the organometallics of lead and tin have similar activities, it is the aim of the present study to compare their activities in the light of chloride independent energy potentiated uncoupling, as discussed in Chapter 3 of this thesis. The organoleads triethyl- and tributyllead acetates have been used for this purpose.

5.2 Materials and Methods

Materials

All chemicals used were of analytical grade. The trialkylleads were obtained from All other sources of chemicals have been listed previously.

Methods

Trialkyllead Solutions

All trialkylleads were added to the assay as dimethylformamide solutions

serially diluted from 10mM stock, kept at room temperature in the dark.

All other methods employed in the present chapter have been described in the methods sections in Chapters 2 and 3.

5.3 Results

The Inhibition of ATP Synthase and Hydrolase Activities

Table 5.i compares the I_{50} values for the inhibition of ATP synthase and hydrolase activities of rat liver mitochondria with triethyl- and tributylleads and tins. Triethyllead is a poor inhibitor of the ATPase complex in both its synthetic and hydrolytic capacities. Indeed, triethyllead at 10 nmoles/mg protein is observed to stimulate ATPase activity. This activity predominates, which is in contrast to triethyltin where 70% inhibition is experienced at 10 nmoles/mg. A maximal two fold stimulation of ATPase activity has been reported in 0.15M chloride medium for both triethyllead and tin (Aldridge et al, 1977), although inhibition follows at concentrations greater than 2 nmoles/mg protein of triethyltin (see Chapter 3). The 10% stimulation experienced on triethyllead addition is consistent with an uncoupler stimulated activity. Further profound differences are observed between the two compounds as triethyllead undergoes total ATP synthase inhibition before significantly affecting ATPase activity; the very opposite of that observed with triethyltin. These effects are presented graphically in Figure 5.i together with those produced by the tributylmetals. Similarly to triethyllead, tributyllead completely inhibits oxidative phosphorylation before halting ATPase activity, although the effect is noticably less marked. Overall, the trialkylleads appear more potent inhibitors of oxidative phosphorylation than the equivalent trialkyltins. However, despite this, their ultimate modes of action have been reported to be similar; clearly, concentration dependent

Table 5.i

The Sensitivities of Mitochondrial ATPase and ATP Synthase to triorganoleads and tins

Trialkylmetal		I_{50} (nmoles/mg protein) ± SD	
		ATPase	ATP synthase
Triethyllead acetate	(3)	18 ± 1.6	1.6 ± 0.4
Triethyltin sulphate	(5)	2.8 ± 0.4	9.8 ± 0.5
Tributyllead acetate	(3)	4.8 ± 0.5	1.0 ± 0.2
Tributyltin acetate	(3)	2.0 ± 0.2	7.8 ± 0.2

Legend:
ATPase and succinate driven ATP synthase activities were estimated as described in the 'materials and methods' of Chapter 3. The numbers in parentheses indicate the numbers of duplicate experiments performed. Control rates of ATPase and ATP synthase ranged between 30-40 and 80-100 nmoles/mg/minute respectively.

Figure 5.i

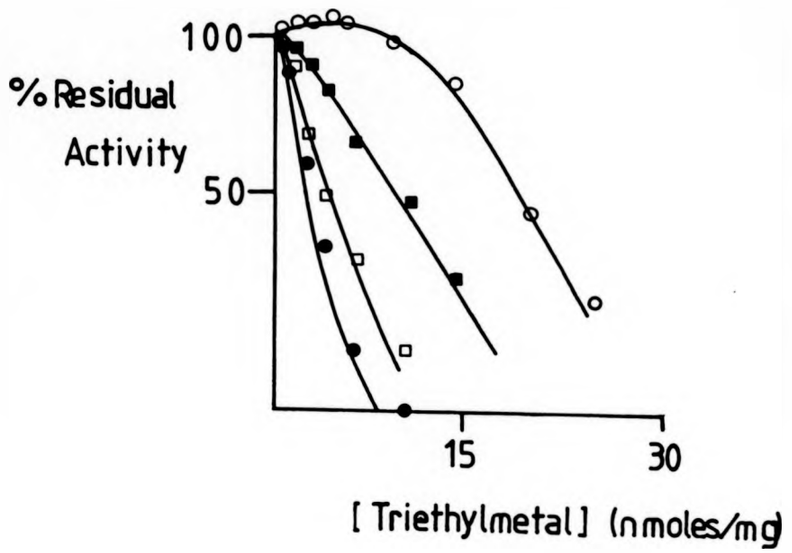
Inhibition of ATP Synthesis and Hydrolysis in Rat Liver Mitochondria by Trialkyl Leads and Tins

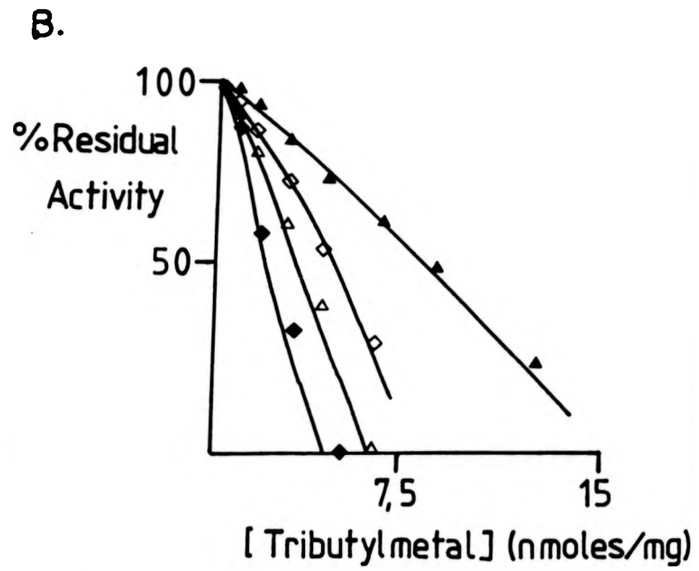
Legend:

A. Titration profiles for triethyllead acetate (● ○) and triethyltin sulphate (■ □).

B. Titration profiles for tributyllead acetate (◆ ◇) and tributyltin acetate (▲ △).

These estimates were performed as described in 'materials and methods' of Chapter 3. The open and hatched symbols are ATPase and ATP synthase respectively, where activities are expressed as percentiles of control values (ATPase 37nmoles/mg/minute, ATP synthesis 73nmoles/mg/minute).





effects are operating.

Trialkylleads as Uncouplers of Mitochondria

Table 5ii demonstrates the relative potencies of the trialkylleads and tins to uncouple, based on the $DSMP^+$ fluorescence assay. The trialkylleads are far more potent uncouplers, reducing $\Delta\Psi$ by +100mV at 4.0 and 2.25 nmoles/mg protein for triethyl and tributyllead respectively. The values are approximately five fold less than those observed with the trialkyltins. Clearly, uncoupling is a major function of trialkylleads on rat liver mitochondria.

Figure 5ii presents the titrations of the tributylleads against the components of proton motive force. Comparison against the equivalent trialkyltins demonstrates the superior activities of the trialkylleads to facilitate energy potentiated uncoupling. These large variations are indicative of the inhibitors having profoundly different principle functions to bring about net inhibition.

5.4 Discussion

The trialkylleads are clearly potent inhibitors of oxidative phosphorylation, but appear to be only moderate inhibitors of ATP hydrolase compared with their trialkyltin counterparts. Such behaviour is inconsistent with a direct interaction with the ATPase complex. The stimulation of ATPase activity at low concentrations of trialkyllead is consistent with an uncoupler stimulated activity releasing the accumulation of protons, albeit low, compared with the chloride dependent stimulation of trialkyltins and that reported for trialkylleads (Aldridge et al, 1977). The effect may be small due to simultaneous antagonism of the ATPase or non-proportional proton mobility. Considering the higher reported activities in the presence of chloride, the latter explanation appears more likely, that is, presupposing the ATPase undergoes no prejudicial interaction with free chloride. Uncoupling should prevent

Table 5.ii

The Relative Potency of Trialkyl- leads and tins on the Membrane Potential of Rat Liver Mitochondria

Trialkylmetal	+ 100mV $\Delta\Psi$ ± SD (nmoles/mg)
Triethyllead acetate	4.0 ± 0.5
Triethyltin sulphate	19.0 ± 2.3
Tributyllead acetate	2.25 ± 0.3
Tributyltin acetate	13.0 ± 2.6

Legend:
The above data is based on $DSMP^+$ fluorescent changes calibrated against the distribution of $[^3H]$-DSMP I as described in Chapter 2. The concentrations represent the mean of 5 individual assays ± standard deviation.

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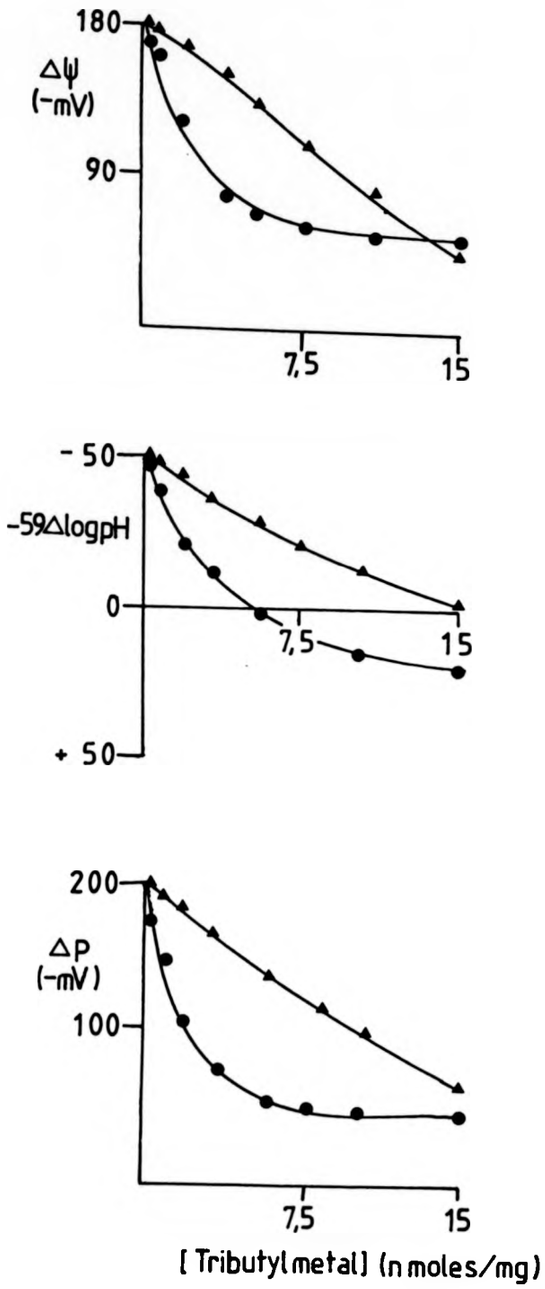
Figure 5.ii

The Effects of Tributyl- Leads and Tins on $\Delta\Psi$, ΔpH and Δp

Legend:
$\Delta\Psi$ and Δp estimates were based on those obtained with $[^3H]$-$TPMP^+$. The distributions of 2μM (1μCi) $[^3H]$-$TPMP^+$ and 25μM (0.5μCi) $[^{14}C]$-lactate were measured as described in 'materials and methods' of Chapters 2 and 3 respectively.
▲ Tributyltin acetate; ● Tributyllead acetate.



ATP synthesis.

This conclusion is substantiated by considering parallel changes in membrane potential and proton motive force. The membrane potential is reduced by +100mV at 2.25 nmoles/mg for tributyllead acetate, which appears sufficient to reduce the rate of ATP synthesis by 60% but still retaining almost control rate ATP hydrolase activity. Tributyllead further halts ATP synthesis at approximately 4.0 nmoles/mg, which produces a correspondingly low net Δp of 80-90mV. This proton motive force should not be capable of driving ATP synthesis in the presence of largely unmodified ATP synthase molecules requiring substantial proton flux. However, under such circumstances ATP hydrolysis should continue unabated.

These effects may be considered as an extreme case of those discussed earlier for DBCT and the very antithesis of those experienced with Ve2283. In general, the trialkyltins appear as moderate to good inhibitors of the ATPase complex in both its capacities; in contrast to the trialkylleads which appear to function as potent uncouplers, independent of chloride/hydroxyl exchange.

5.5 Conclusion

Trialkylleads have been shown to be potent uncouplers of oxidative phosphorylation. They exert their effects via reduction in proton motive force, which appear not to be parallelled by inhibitory actions on the ATPase complex. In the absence of appreciable chloride/hydroxyl exchange the trialkylleads appear to have different functional priorities over those of the majority of trialkyltins.

CHAPTER 6

Energetic Studies on Yeast Mitochondria

6.1 Introduction

It has long been known that isolated yeast mitochondria appear poorly coupled compared to their mammalian counterparts, as ascertained by their lack of stimulation of the F_1 Fo-ATPase activity with uncouplers (Kovac et al, 1968) and lower P/O ratios (Linnane et al, 1962; Vitols et al, 1961; and Mattoon and Balcavage, 1966). However, the membrane bound ATPase activity appears to be oligomycin sensitive, similar to mammalian systems (Kovac, 1968; and Somlo, 1968; see Lloyd, 1974 for review).

Biochemical studies focussed on mutants of the yeast mitochondrial ATPase have revealed that oligomycin resistivity bears no apparent energetic handicap (Griffiths and Houghton, 1974; and Somlo et al, 1974), merely a reduced affinity for the drug (Griffiths and Houghton, 1974). However, studies on the mit^- mutations at the pho 1 locus, closely linked to Oli 2 on mitochondrial DNA (Somlo et al, 1977), have revealed a highly abnormal ATPase complex. The mitochondrial ATPase produced by the mutant exhibits weak membrane linkage, exaggerated activation, an increased sensitivity to oligomycin, dual behaviour on purification and low rates of oxidative phosphorylation in isolated mitochondria (Somlo et al, 1977 and 1979).

It is the purpose of the following chapter to determine the energetic properties of yeast mitochondrial preparations from various mutant strains, despite their inherently 'leaky' nature. The current study is focussed on the ability of yeast to generate a mitochondrial membrane potential, as ascertained by the fluorescent probes $DSMP^+$ and $DSEP^+$, under various inhibitory conditions.

Similar studies have been reported on whole yeast cells using the cyanine

dye 3,3'- dipropylthiocarbocyanine iodide as a fluorescence indicator (Kovac and Varecka, 1981). The fall in the steady-state fluorescence of the dye was attributed to a depolarisation of membrane potentials, the major contribution of which was provided by the mitochondrial membrane potential as indicated by specific uncouplers and pore-forming agents. However, a certain proportion was assigned to the plasma membrane which remained inducible in petites by the addition of metabolites.

6.2 Materials and Methods

Materials

Sorbitol was purchased from Fisons. Nyastatin was purchased from Serva and '1799' (1,1 - bis (hexafluoroacetonyl) acetone) was obtained from Dow Chemical Corporation Inc. Zymolyase was purchased from Miles. All other reagents have been described in earlier chapters.

Yeast Strains

The yeast strains employed in Chapter 6 and 7 are listed in Table 6.i together with their genotypes and origin.

Culture Media

Basically, three types of complex media were used: YPD, YPG and YPGal.

YPD	YPG	YPGal
2% (W/V) D - Glucose	3% (V/V Glycerol	3% (W/V) Galactose
1% (W/V) Yeast Extract	1% (W/V) Yeast Extract	1% (W/V) Yeast Extract
1% (W/V) Bactopeptone	1% (W/V) Bactopeptone	1% (W/V) Bactopeptone

When solid media was required 2.3% (W/V) agar was added prior to autoclaving.

Antibiotic Media

Antibiotics were always added to the media after autoclaving and cooling. For solid media the antibiotics were added before pouring of the plates at 45-50^{o} C. All antibiotics were added in ethanolic solution except triethyltin sulphate (TET) which was added in aqueous solution.

Table 6.i

Strains of S. cerevisiae

Name	Genotype			
	Nuclear	Mitochondrial	Ant^R	Origin
D273/10BA1	α met	ρ^+	-	A. Tzagoloff
D22	a ade	ρ^+	-	D. Wilkie
DS14	α met	ρ^- Oli^R2-118	-	Macino and Tzagoloff, 1980
D27/76	α met	ρ^+ Oli^R2-76	Oli^R	Lancashire and Mattoon, 1979
D27/A118	α met	ρ^+ Oli^R2-118	Oli^R	Meiosis of CDS14 x D27 Diploid
D27/110	α met	ρ^+ Oli^R1-110	Oli^R	Lancashire and Mattoon, 1979
D27/92	α met	ρ^+ Oss^R1-92	Oss^R	Lancashire and Mattoon, 1979
D27/101	α met	ρ^+ Oss^R2-101	Oss^R	Lancashire and Mattoon, 1979
M5-16	α lys	ρ^+ mit^-oxi 3	-	Connerton et al, 1984
M28-81	α lys	ρ^+ mit^-pho 1	-	Connerton et al, 1984
mit175	α ura	ρ^+ mit^-mit175	-	Bolotin-Fukuhara et al, 1977
M17-231	α lys	ρ^+ mit^-cob 1	-	Connerton et al, 1984
D22/69	a ade	ρ^+	Ven^R	Lancashire and Griffiths, 1975b
D22/71	a ade	ρ^+	$R6G^R$	Carignani et al, 1977
D22/EC6	a ade	ρ^+	TET^R	Lancashire and Griffiths, 1975a

Methods

Sterilisation

Pipettes were sterilised by dry heat at 150°C overnight. All other sterilisations were performed by autoclaving at 15 psi for 20 minutes at 160°C.

Preparation of Agar Slopes

Bijou bottles were filled to approximately half capacity with YPD plus 2.3% agar No.3, loosely capped and sterilised. After cooling in inclined racks, the slopes were allowed to dry before tightening the caps and storing at 4°C.

Temperature

All incubations were performed at 29±1°C, unless otherwise stated.

Maintenance of Strains

Slopes were sub-cultured every six months and incubated for 2-3 days before storage at 4°C. All drug resistant strains were tested with various concentrations of antibiotics before use TET (5,10,20 µg/ml), R6G (25, 50, 100 µg/ml), venturicidin (0.5, 1, 2 µg/ml), oligomycin (0.5, 1, 2µg/ml), and ossamycin (1, 2, 4µg/ml).

Growth of Yeast

Batch cultures of yeast were grown to late log phase in YPG from pre-culture, by forced aeration of 500ml aliquots in 2 litre fluted Erlenmyer flasks. The yeast were harvested in 250ml centrifuge bottles at 2,000 x g for 10 minutes. The pelleted cells were washed in cold sterilised distilled water before suspension in breaking buffer (0.5M Sorbitol, 10mM Tris/HCl pH 7.5, 1mM EDTA, 0.1% BSA) at 0.5ml per gramme of wet cells.

Preparation of the Mitochondrial Fraction

All the following operations were carried out at 0-4° C. The cell suspension was placed in a stoppered glass bottle with a half volume

of ballotini (0.4-0.5mm diameter) and broken by the hand-shaking method of Lang et al, (1977), in 3 x 2 minute cycles with 1 minute intervals on ice. The broken cell supernatant was decanted and the residual glass beads washed thoroughly with breaking buffer before pelleting the unbroken cells and cell debris at 2000 x g for 15 minutes. The supernatant from this spin was then transferred to 60ml polycarbonate centrifuge tubes and the mitochondrial fraction isolated by spinning at 16000 x g for 30 minutes. This pellet was suspended in breaking buffer minus BSA at 30mg/ml.

The mitochondrial suspension produced in this manner was then layered onto a discontinuous sucrose gradient (14ml each of 15%, 30%, 50% and 70% (W/V) sucrose solutions containing 10% methanol, 10mM Tris/HCl pH 7.5, 1mM EDTA) and centrifuged in a Beckman 42.1 Ti rotor at 28 K r.p.m. for 3-4 hours. The mitochondrial band was visually collected at an approximate density of 1.20g/cm^3 and diluted into breaking buffer minus BSA to 20mg/ml.

When small quantities of mitochondria were required from various strains, the cells were broken enzymatically by digestion with Zymolyase 2,000 in 1M sorbitol, 10mM Tris/HCl pH 7.5, 1mM EDTA 30^{o}C for 1 hour. The resulting spheroplasts were then lysed osmotically by diluting the suspension mix to 0.6M sorbitol; the mitochondrial fraction was collected as above.

Fluorescence Monitoring of Mitochondrial Membrane Potential

Yeast cells were spheroplasted as above with Zymolyase and pelleted and washed in 1M sorbitol, 10mM Tris/HCl pH 7.5, 1mM EDTA before keeping on ice. The spheroplasts were diluted into 250mM mannitol, 10mM Tris/HCl pH 7.5 in the fluorescence cuvette and immediately placed in the fluorimeter with overhead stirring before the addition of $DSMP^+$ or $DSEP^+$ at 1 μM. The fluorescence signal was allowed to achieve steady-state

before the addition of inhibitors or exogenous substrates, invariably the *in situ* liberated mitochondrial membranes appeared energised from wild type cells.

6.3 Results and Discussion

In Vivo Drug Resistance

The minimum inhibitory concentrations for the various mutant strains of yeast are presented with their wild type progenators D27 (a subclone of the α met strain D273/10BA1) and D27 in Table 6ii. The majority of drug resistant mutations isolated show cross resistance to other mitochondrially directed drugs, in particular those selected for R6G, TET and venturicidin resistance. However, specific drug resistant alleles may be selected for *Oli 2* and *Oss 1* mutations, Oli^R 2-76 and Oss^R1-92 respectively, for further molecular analyses. The mutations conferring specific drug resistance phenotypes are exceptions rather than the rule, in particular, those distinguishing resistance to oligomycin and ossamycin, suggesting that the sites of action of these drugs may overlap. A previous study in this laboratory, Griffiths and Houghton, (1974), failed to make this distinction in some forty independent drug resistant isolates examined. This conclusion is further exemplified by considering the *Oli 2* mutation Oli^R2-118 of Macino and Tzagoloff, (1980), which appears to show cross-resistance to ossamycin and has been shown to be a point mutation at residue number 254 in the Oli.2/ subunit 6 coding sequence, as ascertained by DNA sequence analysis. However, these general conclusions must be validated by checking their resistance types at the mitochondrial level *in vitro*.

In Vitro Drug Resistance

Mitochondria were isolated from the mutant strains as indicated in the above paragraphs, chosen on the basis of their *in vivo* behaviour. ATPase assays were performed as described earlier and yielded rates

Table 6.ii

Inhibitor Tolerances of Mutant Strains

Strain	Mutation	Tolerance to Inhibitor (μg/ml)			
		Oligomycin	Ossamycin	Venturicidin	Triethyltin
D273/10BA1	Wild type	0.1	0.5	0.1	1.0
D27/76	$Oli^R 2\text{-}76$	1.0	0.5	0.1	1.0
D27/A118	$Oli^R 2\text{-}118$	>10	>10	0.1	1.0
D27/110	$Oli^R 1\text{-}110$	>10	>10	0.1	1.0
D27/92	$Oss^R 1\text{-}92$	0.1	5.0	0.1	1.0
D27/101	$Oss^R 2\text{-}101$	1.0	>10	0.1	1.0
D22	Wild type	0.1	0.5	0.1	1.0
D22/69	Ven^R	>10	>10	>10	>10
D22/72	$R6G^R$	>10	>10	>10	>10
D22/EC6	TET^R	>10	>10	>10	>10

of ATP hydrolysis in the range of 4-5 μmoles/mg/minute from wild type and drug resistant mitochondrial preparations. In addition to the drug resistant mutants surveyed are a series of mit$^-$ mutations spanning the Oli 2 region presented for comparison as I_{50} values in Table 6.iii. The mit$^-$ mutation pho 1 demonstrated a reduced activity of 1.0 μmoles/mg/minute which is expected since it has been located within the Oli 2 gene (Connerton et al, 1984 and Somlo et al, 1985), coding for a 20 K Dalton subunit of mitochondrial ATPase (Roberts et al, 1979). The nature of the residual ATPase activity of the leaky pho 1 mutant is due to a heterogenous population of ATPase molecules (Somlo et al, 1977; and Somlo and Krupa, 1979), created due to a frameshift in the Oli 2 gene and its partial compensation (Somlo et al, 1985).

The sole resistance phenotypes of the mutations Oli^{R}2-76 and Oss^{R}1-92 is further verified at the level of the ATPase complex with the information that the I_{50} values (see Table 6.iii) demonstrate that each allele causes at least a 5 fold increase over the wild type progenator for their respective drugs, but still maintain wild type sensitivities for other inhibitors of oxidative phosphorylation. These rare mutations are well sited for further molecular analysis (for further information see Chapter 7). The Oli 2 mutation of Macino and Tzagoloff, (1980), Oli^{R}2-118, also bears out its cross resistance in vitro, demonstrating I_{50} values some 7 and 8 fold over wild type for oligomycin and ossamycin respectively. It is noted that this allele also produces an I_{50} value for venturicidin which is double that of the wild type, the drug binding sites may overlap. This conclusion is further substantiated by considering the wide cross-resistance imposed by the Oli 1 allele Oli^{R}1-110, the Oli 1 gene, subunit 9, may play a central role in the binding of many drugs affecting ATPase functions by interaction with the Fo moiety of mitochondrial ATPase. The Oss 2 allele, Oss^R 2-101, presumably

Table 6.iii

Inhibition of Mitochondrial ATPase by Various Inhibitors

Strain	Mutation	I_{50} Values (µg/mg protein)			
		Oligomycin	Ossamycin	Venturicidin	Triethyltin
D273/10BA1	Wild type	3.0	3.0	0.4	0.8
D27/76	Oli^R2-76	17.5	3.0	0.3	0.6
D27/A118	Oli^R2-118	19.5	25.0	0.7	0.8
D27/110	Oli^R1-110	19.0	22.0	0.8	0.9
D27/92	Oss^R1-92	3.0	15.0	0.4	0.8
D27/101	Oss^R2-101	4.5	9.5	0.5	0.8
M5-16	mit^-oxi 3	3.0	3.0	0.4	0.8
M28-81	mit^-pho 1	1.5	2.0	0.4	0.6
mit175	mit^-mit175	2.5	3.0	0.3	0.8
M17-231	mit^-Cob 1	3.0	3.0	0.4	0.8
D22	Wild type	2.5	2.0	0.2	0.2
D22/69	Ven^R	4.0	4.0	1.5	1.5
D22/72	$R6G^R$	4.0	4.0	1.0	1.5
D22/EC6	TET^R	4.5	4.0	1.5	1.5

also centred on the subunit 9 gene, shows cross-resistance, although not to the same degree.

The mit$^-$ mutations directed at cytochrome oxidase and apocytochrome b have little effect on their respective sensitivities to the drugs directed at OS-ATPase. The pho 1 mutation shows a higher sensitivity to the drugs than the wild type, possibly due to an altered ATPase. The mit$^-$ mutation mit 175, despite mapping in the Oli 2 region (Connerton et al, 1984), shows little deviation from wild type in respect of its sensitivities to drugs directed at mitochondrial ATPase. The nature of this mutation remains obscure.

The strains based on the wild type D22 and selected for antibiotic resistance to venturicidin, rhodamine 6G and triethyltin show apparently cohesive behaviour, since all show wide cross-resistance in vivo but only slightly raised resistance phenotype in vitro. These mutations may not be centred on specific genes whose products are structural proteins of mitochondria.

Monitoring of Mitochondrial Membrane Potential in Yeast

Figure 6.i presents a series of fluorescence traces obtained from in situ lysed wild type yeast protoplasts (D273/10B A1) in the presence of 1μM of the fluorescence dye DSMP$^+$ (Excitation 479nm Emission 589nm) and DSEP$^+$ (Excitation 479nm-Emission 582nm). After the initial burst of fluorescence due to non-specific emission, both dyes are observed to undergo a steady increase in fluorescent intensity which eventually stabilises after 3-5 minutes (see Figure 6.ia and b for DSMP$^+$ and DSEP$^+$ respectively). The second slower build-up of fluorescence is sensitive to CCCP and titratable with tributyltin oxide. This behaviour resembles that of rat liver mitochondria studied in previous chapters of this thesis. Further evidence as to the nature of the fluorescence signals is provided by its sensitivity to potassium ions in the presence

Figure 6.i DSEP$^+$ Mediated Membrane Potential Dependent Fluorescent Changes of *in situ* Lysed Yeast Spheroplasts

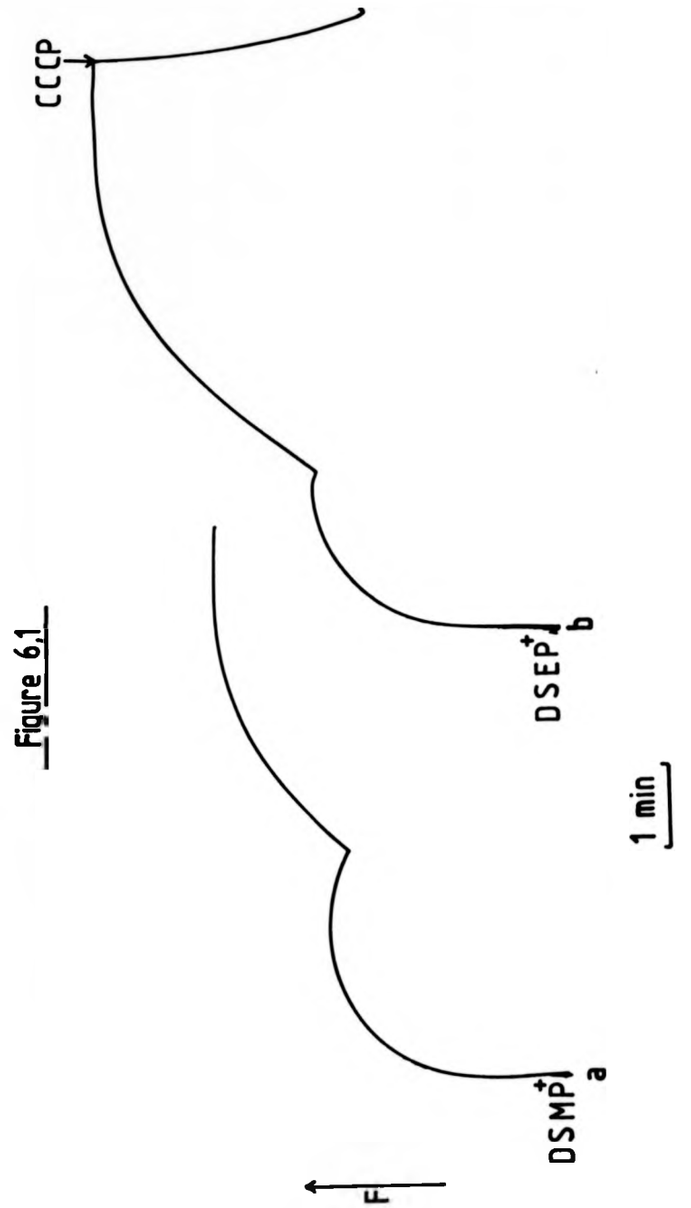


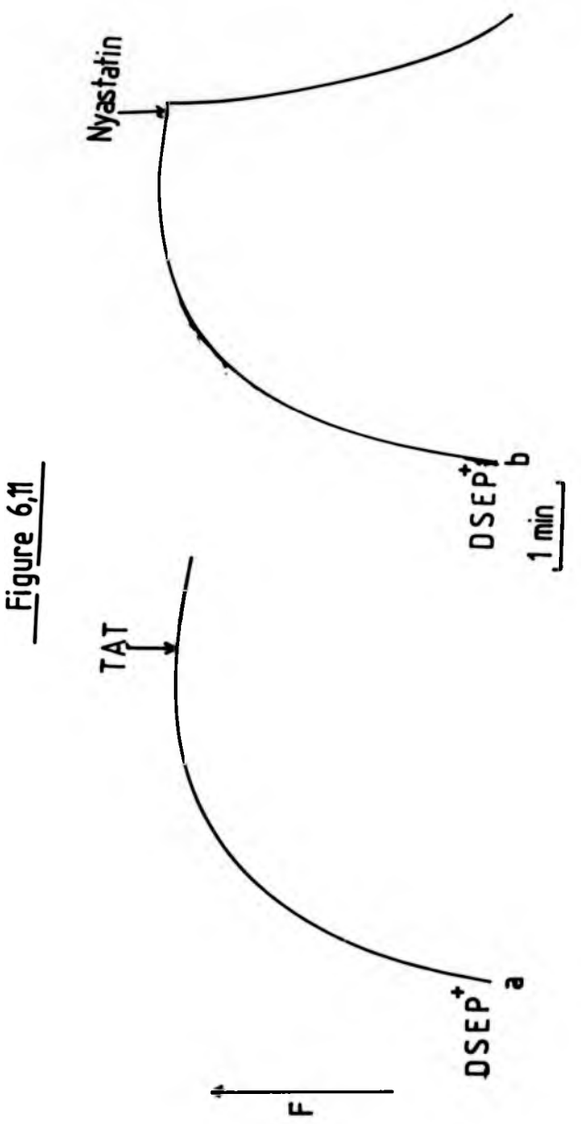
Figure 6.1

of valinomycin. This is strong evidence that the trialkyltin titratable fluorescence is the result of endogenous mitochondrial membrane potentials, since Saccharomyces cerevisiae is not prevented from growth on glucose by valinomycin but only on non-fermentable carbon sources, indicating the antibiotic's vital action is centred on the mitochondrial oxidative process. As a consequence of the yeast's insensitivity to valinomycin/K^+ gradients under fermentative growth, Kovac and Varecka, (1981), demonstrated that neither whole cells nor protoplasts are affected in the cyanine dye based system by 0.1M KCl in the presence of valinomycin.

The styryl dyes have little effect on whole yeast cells other than an intrinsic increase in fluorescence due to the turbidity of the suspension and light scattering, this is in contrast to the cyanine dye reported by Kovac and Varecka, (1981). Neither do osmotically stable spheroplasts produce fluorescent signals which respond to the specific uncouplers of mitochondrial membrane potential. However, a somewhat depressed response is observed with the general pore forming agent nyastatin, possibly due to a partition of the dye across the plasma membrane (see Figure 6.ii a and b). It is interesting to note that at this point the dye $DSEP^+$ gives a better signal to noise ratio than its $DSMP^+$ counterpart. $DSEP^+$ produces much less intrinsic fluorescence on addition to turbid material, and as such is more suitable for the application of the above technique, where relatively dense suspensions of lysed protoplasts are employed.

Inhibitors of the respiratory chain also cause a marked drop in membrane potential. Figure 6.iii demonstrates the effects of rotenone, antimycin A and cyanide in reducing membrane potential via the inhibition of complexes I, III and IV respectively. The fall in membrane potential initiated by the respiratory chain inhibitors is less marked than

Figure 6.ii Changes in $DSEP^+$ Fluorescence with Intact Spheroplasts



the actions of the uncouplers presented above. This is possibly due to the presence of residual metabolites liberated on protoplast lysis and/or the endogenous intra-mitochondrial ATP level of the mitochondria so liberated. Additions of exogenous succinate (5mM) after inhibition by rotenone certainly suppresses the fall in potential.

The mitochondrial ATPase inhibitors oligomycin, ossamycin and venturicidin had little effect on the fluorescence level of $DSEP^+$. Despite inhibition of complex V the membrane potential remains unchanged. However, the inhibitor DCCD which will affect ATPase demonstrates a modest fall in membrane potential as monitored by $DSEP^+$. This may result from other DCCD mitochondrial interactions, in particular with cytochrome oxidase (complex IV).

Mutant Mitochondrial Functions

Table 6.iv summarises the application of the above technique to the mutant strains together with their respective ATPase activities. The mutations' origins have been described in Table 6.i in the 'Materials and Methods' section of this chapter, but basically they fall into four categories. The first is represented by DS14, a petite strain deficient in most respiratory functions. The strains had accordingly no mitochondrial membrane potential as detectable by sensitivity to the uncouplers CCCP, triethyltin, '1799' and valimomycin mediated potassium uptake, nor oligomycin sensitive ATPase activity. The second group are those mutations which are bioenergetically competent but possess bona fide mitochondrially located drug resistant mutations. As described earlier in this chapter, they display their corresponding resistant and cross-resistant phenotypes at the level of the ATPase. All have wild type mitochondrial potentials and sensitivities to uncouplers, further evidence to their lesions solely conferring an inability to bind the respective drugs efficiently.

Table 6.iv

Legend

The properties of the mutant mitochondria are summarised in terms of their resistance (R) or sensitivities (S) to various drugs. The ATPase activities and fluorescence signals are scored as three crosses (+++) for wild type properties, two crosses (++) significantly diminished (>50%), and one cross (+) for residual activity (>10%).

Table 6.iv

The In Vitro Activities of Mitochondrially Directed Mutations of S. cerevisiae

	ATPase	Oli	Oss	Ven	TET	Δψ	Oli	Oss	Ven	TET	1799	Val/K^+	CCCP
Wild type	+++	S	S	S	S	+++	R	R	R	S	S	S	S
Class 1													
ρ^- Oli^R2-118	+	R	R	R	R		R	R	R	R	R	R	S
Class 2													
ρ^+ Oli^R2-76	+++	R	S	S	S	+++	R	R	R	S	S	S	S
ρ^+ Oli^R2-118	+++	R	R	R	S	+++	R	R	R	S	S	S	S
ρ^+ Oli^R1-110	+++	R	R	R	S	+++	R	R	R	S	S	S	S
ρ^+ Oss^R1-92	+++	S	R	S	S	+++	R	R	R	S	S	S	S
ρ^+ Oss^R2-101	+++	<S	R	S	S	+++	R	R	R	S	S	S	S
Class 3													
ρ^+ mit$^-$oxi 3	+++	S	S	S	S		R	R	R	R	R	R	S
ρ^+ mit$^-$pho 1	+	S	S	S	S	++	R	R	R	S	S	S	S
ρ^+ mit$^-$mit175	++	S	S	S	S		R	R	R	R	R	R	S
ρ^+ mit$^-$cob 1	+++	S	S	S	S		R	R	R	R	R	R	S
Class 4													
ρ^+ TET^R	+++	S	S	S	<S	+++	R	R	R	S	S	S	S
ρ^+ $R6G^R$	+++	S	S	S	S	+++	R	R	R	S	S	S	S
ρ^+ Ven^R	+++	S	S	S	S	+++	R	R	R	S	S	S	S

The third class of mutation are the mit⁻ mutations having specific lesions in mitochondrial genes, those of Oxi 3 (encoding the large subunit of cytochrome oxidase), Cob (encoding apocytochrome b), pho 1 (encoding subunit 6 of OS-ATPase) and mit175 (of unknown function in the Oli 2 region). All the mutations as described above have oligomycin sensitive ATPase activities (however, pho 1 has a particularly sensitive enzyme complex), however, those mutations affecting structural elements of the respiratory chain produce depressed fluorescent responses which are insensitive to most uncouplers. The exception being CCCP which appears to command a more wide ranging effect. The pho 1 mutation displays expected wild type sensitivities to the uncouplers, however, the mutation mit175 mapping in a similar region of the genome (Connerton et al, 1984), behaves as those mutations in the genes of electron transport complexes. This mutation displays only a residual membrane potential under these conditions which is sensitive to CCCP, but is insensitive to triethyltin, '1799' and valinomycin mediated potassium diffusion. This evidence suggests mit175 is deficient in an element of the electron transport machinery, a fact born out by an aberrant cytochrome spectra (see the following chapter for further information).

The fourth and final class of mutation employed in this study are those mutants selected as drug resistant to the mitochondrial directed inhibitor triethyltin, rhodamine 6G and venturicidin, but whose genetic location is not mitochondrial. In general, these mutations despite requiring 10-40 fold drug concentrations to affect growth in vivo, do not have corresponding in vitro activities, neither as an insensitive ATPase to triethyltin or venturicidin, or an insensitivity to the effects of triethyltin on membrane potential. These mutations are essentially wild type in their in vitro behaviour. This data must lead to the conclusion that the mutations are not in genes specifying

structural elements of mitochondria. The mutations may affect their resistance in either more efficient detoxification or permeation of the cell membrane.

6.4 Conclusion

A qualitative assay of mitochondrial membrane potential has been developed in the yeast Saccharomyces cerevisiae based on the in situ lysis of protoplasts in the presence of the fluorescent styryl dye $DSEP^+$. The use of $DSEP^+$ in this application has proved to be more favourable over that of its analogue $DSMP^+$, due to inherent reduction in the signal to noise ratio of the fluorescence. The assay system has been shown to respond to uncouplers of mitochondrial membrane potential in a specific manner, in particular with the imposition of a potassium diffusion gradient in the presence of valinomycin which is known only to affect yeast during oxidative growth.

An attempt has been made to coordinate the in vivo and in vitro modes of action of yeast mutations directed at mitochondrial functions. These studies have been based on the mutations' abilities to effect mitochondrial drug sensitive ATPase activity and uncoupler sensitive membrane potential. Attention has also been focussed on both in vivo and in vitro cross-resistant drug mutations, despite having a single mitochondrial locus. The inhibitory sites of ATPase/synthase inhibitors oligomycin, ossamycin and venturicidin may overlap.

Chapter 7

DNA Sequence Analysis of the Oli 2 Region of the mt-Genome of S.cerevisiae

7.1 Introduction

The following chapter is aimed at elucidating the aminoacid changes in the Oli 2 region of yeast mitochondrial DNA (mt DNA) responsible for the resistance to oligomycin and ossamycin. The alleles chosen for this study are Oli^R 2-76 and Oss^R 1-92, which are unique mutations showing no cross-resistance, both in vivo (Lancashire and Matoon, 1979), and in vitro (see Chapter 6 of this thesis). Such data is consistent with the mutation's loss of affinity for the drugs at, or close to, their specific binding sites. Further localisation of the drug resistant alleles was accomplished by five structure genetic mapping, using the technique of petite deletion mapping (Connerton et al, 1984). The starting grande (ρ^+) for this study was a double mutant containing both the Oli 2-76 and Oss 1-92 alleles and as such was chosen for subsequent molecular analysis. DNA sequence data between the Oxi 3 gene (subunit 1 of cytochrome oxidase) and beyond the end of the Oli 2 gene will be presented in this chapter (see Figure 7.i, and Dujon, 1982 for genome organisation), including the recently reported subunit 8 sequence (or Aap 1 gene) and putative maturase (Seraphin et al, 1985). Corroborating data will also be presented as to the nature of the drug resistant alleles as single point mutations in the strains D27/76 and D27/92 for oligomycin and ossamycin respectively. (For further information see the following introductory paper).

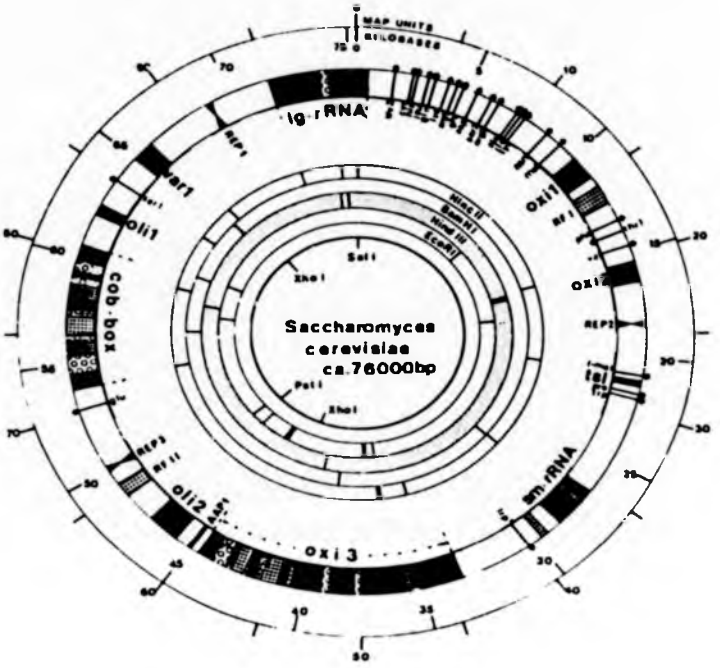


Figure 7.1 Physical and genetic map of the mitochondrial genome of the yeast *Saccharomyces cerevisiae*. The map has been taken from Dujon (1983). Restriction map (inner circles) shown here is from the "long strain" (KL14-4A). For comparison with the short strain, see Morimoto & Rabinowitz (1979). The genes have been placed on the genetic map (outer circle) on scale with respect to restriction sites. The references for various genes have been given in the text. ■ exons, ▦ open reading frames, ▦ introns.

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MGG

Genetics of Oxidative Phosphorylation: Petite Deletion Mapping of the *Oli 2* Region of the Mitochondrial Genome of *Saccharomyces cerevisiae*

I.F. Connerton, M.K. Ray, W.E. Lancashire*, and D.E. Griffiths
Department of Chemistry and Molecular Science, University of Warwick, Coventry CV4 7AL, U.K.

Summary. Petite deletion mapping has been carried out for the *Oli 2* region of the mitochondrial genome of *Saccharomyces cerevisiae* to produce a fine structure genetic map. Previously unlocated *mit*⁻ mutants together with the drug resistant loci *Oli 2* and *Oss 1* have been ordered between the cytochrome oxidase and apocytochrome b genes.

As a result of this study a series of isogenic ρ^- clones have been isolated spanning the *Oli 2* region.

Introduction

Two regions of the mitochondrial genome of *Saccharomyces cerevisiae* have been associated with mitochondrially synthesised subunits of oligomycin-sensitive adenosine triphosphatase (ATPase). The regions were originally defined as two genetically unlinked loci conferring oligomycin resistance, *Oli 1* and *Oli 2* (Avner and Griffiths 1973; Avner et al. 1973).

The *Oli 1* region was further defined by genetically linked venturicidin and ossamycin resistant mutants, *Ven* and *Oss 2*, together with two more oligomycin resistant loci, *Oli 3* and *Oli 5* (Lancashire and Griffiths 1975a; Lancashire and Mattoon 1979a), and the *pho-2* aerobic growth deficient mutants (Foury and Tzagoloff 1976; Coruzzi et al. 1978). The *Oli 2* locus was found to be genetically linked to another weakly oligomycin resistant mutation, *Oli 4* (Clavilier 1976), an ossamycin resistant mutation, *Oss 1* (Lancashire and Mattoon 1979a) and non-allelic aerobic growth deficient mutants, *pho-1* (Foury and Tzagoloff 1976; Coruzzi et al. 1978) and *pho-8, 9* (Darlison and Lancashire 1980).

DNA sequencing studies have confirmed the *Oli 1* gene encodes subunit 9 of the oligomycin-sensitive ATPase (Hensgens et al. 1979; Macino and Tzagoloff 1979a, b) while the *Oli 2* gene has been located in a reading frame specifying a protein of 259 amino acids and 28.25 KD molecular weight (Macino and Tzagoloff 1980). The identification of the *Oli 2* gene product is less obvious since no further amino acid data is available, but 22.00 KD subunit 6 (Tzagloff and Meagher 1972) has been put forward as a candidate and recently re-established as a highly cycloheximide resistant protein of 20.00 KD (Somlo et al. 1982).

Recombination analysis between the *Oli 2*, *Oli 4* and *pho-1* genetic markers has suggested an order of *Oli 2* — *PHO-1* — *Oli 4* (Somlo et al. 1977), similarly the *Oss 1* marker has been located between *Oli 2* and *Oli 4* (Lancashire and Mattoon 1979a). However it has also been noted that the interpretation of recombination data from intralocus and interlocus crosses in this area of the genome is difficult, due to unexpected allele-specific polarity phenomena and map expansion effects (Lancashire and Mattoon 1979a).

The *mit*⁻ mutations have not been physically characterised with reference to known drug resistance mutations and may be with-holding further functional information. This paper therefore describes a fine structure genetic map of the *Oli 2* region including the drug resistant loci *Oli 2*, *Oss 1* and the *mit*⁻ loci, while generating petites suitable for DNA sequence analysis.

Materials and Methods

1. Yeast Strains

The genotypes and origin of the grande and mit⁻ tester strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. The discriminative ρ^- clones presented here are all derived from the starting grande CD 40 by ethidium bromide mutagenesis.

2. Media and Chemicals

The following media were used: NO (1% yeast extract, 1% peptone, 2% glucose, 0.05 M Na, K phosphate buffer, pH 6.25), N3 (as NO but 2% glucose is replaced by 3% glycerol), WO (0.67% yeast nitrogen base without amino acids, 2% glucose), YPD (1% yeast extract, 1% peptone, 2% glucose) and YP10 (1% yeast extract, 1% peptone, 10% glucose). For solid media 2.3% agar was added to the above.

Antibiotic media (N30l and N30s) were prepared by adding methanolic solutions of oligomycin (1.0 µg/ml) and ossamycin (2.0 µg/ml) respectively to N3 medium after autoclaving as described previously (Lancashire and Griffiths 1975b).

Oligomycin was purchased from Sigma and ossamycin was a gift from Dr. H. Schmitz, Bristol-Myers Co.

* *Present Address:* Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

Offprint requests to: D.E. Griffiths

Table 1. Strains of *Saccharomyces cerevisiae*

Strain	Genotype		Origin
	Nuclear	Mitochondrial	
D27	α *met*	ρ^+	Subclone of D273-10B/A1 (A. Tzagoloff)
JC8/AA1	a *leu kar 1*	ρ^0	Lancashire and Mattoon (1979b)
D27/76	α *met*	ρ^+ *oli*^{s}*2-76*	Lancashire and Mattoon (1979a)
CD15	a *leu kar 1*	ρ^+ *oss*^{s}*1-92*	Lancashire and Mattoon (1979a)
DB1	*a leu kar 1* + / α + + *met*	ρ^+ *oss*^{s}*1-92, oli*^{s}*2-76*	JC8/AA1 × D27/76
DB1-1C	α *met*	ρ^+ *oss*^{s}*1-92, oli*^{s}*2-76*	Meiosis of DB1
CD40	a *leu kar 1*	ρ^+ *oss*^{s}*1-92, oli*^{s}*2-76*	Cytoductant from cross JC8/AA1 × DB-1C
CD24	α *his kar 1*	ρ^+ *mit*$^-$ *pho-9*	Darlison and Lancashire (1980)
CD41	α *his kar 1*	ρ^+ *mit*$^-$ *pho-8*	Darlison and Lancashire (1980)
mit 175	α *ura*	ρ^+ *mit*$^-$	Bolotin-Fukuhara et al. (1977)
M28-81	α *met*	ρ^+ *mit*$^-$ *pho-1*	Foury and Tzagoloff (1976)
M6-239/L	α *lys 2*	ρ^+ *mit*$^-$ *pho-1*	Darlison and Lancashire (1980)
M17-231	α *met*	ρ^+ *mit*$^-$ *cob-1*	Tzagoloff et al. (1976)
M17-162	α *met*	ρ^+ *mit*$^-$ *cob-2*	Tzagoloff et al. (1976)
M9-3	α *proto*	ρ^+ *mit*$^-$ *oxi-2*	Slonimski and Tzagoloff (1976)
M9-3/L	α *lys 2*	ρ^+ *mit*$^-$ *oxi-2*	Selection for lysine auxotrophy
M9-94	α *proto*	ρ^+ *mit*$^-$ *oxi-1*	Slonimski and Tzagoloff (1976)
M9-94L	α *lys 2*	ρ^+ *mit*$^-$ *oxi-1*	Selection for lysine auxotrophy
M5-16	α *proto*	ρ^+ *mit*$^-$ *oxi-3*	Slonimski and Tzagoloff (1976)
M5-16/L	α *lys 2*	ρ^+ *mit*$^-$ *oxi-3*	Selection for lysine auxotrophy

3. Ethidium Bromide Mutagenesis

The grande CD40 was grown to late-log phase in liquid YPD before dilution into YPD plus 10 μg/ml ethidium bromide and incubated with shaking in the dark for 24 h at 29° C. After which the culture was further diluted into YPD plus 20 μg/ml ethidium bromide and incubated again. Samples were withdrawn from the culture after 4 and 8 h and either washed and plated on solid YPD or subjected to further sequential treatment with ethidium bromide.

4. Screening of ρ^- clones

The screening of ρ^- clones was performed as essentially described in 'Procedure A' by Carignani et al. 1979.

5. Restoration Test of mit$^-$ mutants by ρ^- clones

The *mit*$^-$ strains of the senitive strain D27 were grown to late log phase in either YP10 or liquid YPD and a cell suspension spread onto WO plates (2×10^7 cells). ρ^- clones were then cross-replica plated from NO master plates to the tester lawn. The mating plates were incubated for 3 days at 29° C. The resulting diploid growth was then velveteen replicated onto N3, N301 and N30s plates and growth scored after 3 days at 29° C.

Results and Discussion

In this study over 1,000 ρ^- clones were screened for their retention and discrimination of genetic markers directed towards the *Oli 2* region of the mitochondrial genome. Representatives of the classes of ρ^- clones produced in this study are presented in Table 2, showing the *mit*$^-$ × ρ^+ and *ant*^{s} × ρ^- restoration crosses in matrix form. The primary ρ^- clones were designated LP, IC, FC and MR, the products of four independent ehtidium bromide treatments of the starting grande CD40 and a digit (from 1 to 9), then by preceding the digit with an appropriate letter in alphabetical order (eg. IC1 – IC9, ICA1 – ICA9, ICB1 – etc.). Secondary and tertiary clones were further designated by second and third digits respectively. These strains are in general based on the short mitochondrial genome of D273-10B/A1 with the exception of *mit 175* which is derived from the grande FF1210-6C (Bolotin – Fukuhara et al. 1977). The grande CD40, *mit*$^-$ strains CD24 and CD41 were constructed by cytoduction to possess defined nuclear genomes of opposite mating types (Lancashire and Mattoon 1979b).

The coretention and codeletion of the markers in the ρ^- clones is consistant with the assumption that none of the ρ^- mutants presented here have doubly deleted or genetically rearranged genomes.

Approximately 26% of the total ρ^- clones screened had detectable functional genetic information. Of these 75% complemented the *mit*$^-$ testers M9-3/L, M9-94/L and M5-16/L deficient in cytochrome oxidase activity (Slonimski and Tzagoloff 1976). The remaining 25% appear to retain equally portions of the *COB-box* and *Oli 2* regions, as ascertained by the restoration of oxidative growth and drug resistivity in mutants deficient in cytochrome b or directed at oligomycin-sensitive ATPase function.

The coretention and codeletion data in petites allow a map order to be deduced for the markers as summarised in Fig. 1. The *pho-8* and *pho-9* mutations are not allelic with the *pho-1* mutations in M28-81 and M6-239 (Darlison and Lancashire 1980), the two groups are in fact separated by the *Oli 2* and *Oss 1* resistance loci. Petites descriminating the *pho-8* and *pho-9* mutations have also been isolated, indicating these alleles are not homoallelic and indeed have been observed to undergo partial complementation in *pho-8* × *pho-9* crosses on oxidative media (Connerton, I.F., unpublished results).

It is most likely that the resistance loci lie in the *Oli 2* coding sequence which probably codes for subunit 6 of the oligomycin-sensitive ATPase. The *pho-1* mutations are also likely to be within this structural gene.

The location of the *pho-8* and *pho-9* mutations is quite open to speculation. These mutations lie on the *Oxi 3* side of *Oli 2* and they are readily descriminated from the *oli 2* mutations in petites and may be located in a different gene. Sequencing data upstream of *Oli 2* (Linnane, A.W., personal communication) has revealed a putative reading frame of 144 base pairs which has been suggested to be associated with an apparent 10.00 KD protein observed on gels and named *AAP1*. This gene could therefore be the location for the *pho-8* and *pho-9* mutations. Alternatively these mutations could be located in the 5′ flanking region of the *Oli 2* gene and therefore in a controlling region(s). Clearly it is important to rationalise these alternaives, and sequencing of petite and grande DNA is presently underway to accomplish this.

In order to understand any controls employed within the mitochondrial genome it is important to be able to identify deficient mutants both within and out of the coding

Table 2. The Restoration Matrix for the Oli 2 Region

ρ^- (a)	ρ^+ mit$^-$ (α)											
	M9-3/L	M9-94/L	M5-16/L	CD-41	CD-24	D27-Oli^s	D27-Oss^s	M28-81	M6-239/L	mit 175	M17-231	M17-162
CD-40 IC5	***	***	***	***	***	***	***	**	**	**	***	***
CD-40 LPB42				**	**	***	***	**	**	**	***	***
CD-40 IC93						***	***	*	*	*	***	***
CD-40 ICA42						***	***	5c	3c	3c	***	***
CD-40 IC931						***	***	*	*	1c	***	
CD-40 ICA424						***	***	2c	*			
CD-40 IC93A5						**	**					
CD-40 ICB2											***	***
CD-40 ICB28												***
CD-40 ICB9	***	***	**								***	***
CD-40 ICD4	***	***	*						4c	2c	**	***
CD-40IC5A8									*	5c	**	***
CD-40 ICD9	**	***	*									
CD-40 ICB93		**	*									
CD-40 IC2	***	***	*	*	*	***	***	3c	*			
CD-40 ICB4	***	***	***	*	**	***	***	1c				
CD-40 IC5A6	***	***	***	**	**	***	***	**				
CD-40 IC2B1			***	*	*	***	***	*	**			
CD-40 IC2C4			***	*	**	***	***	5c				
CD-40 IC2E9			***	**	**	***	***	*				
CD-40 FCD6			***	NT	**	***	***					
CD-40 LPA1D1				NT	**	***	***	*	*	**		
CD-40 FCD62				NT	**	***	***					
CD-40 MR53				*	*	***	***					
CD-40 LP81					*	***	***					
CD-40 MR6				**	**	***						
CD-40 MR66					***	***						
CD-40 IC2F96	**											
CD-40 LPA12					**							

Scoring of the diploids on non-fermentable substrate:
*** = full and confluent growth of diploids
** = full but not confluent growth of diploids
* = full growth but less than 10 diploid colonies
nc = number of diploid colonies of 5 or less
No mark = no growth
NT = no test

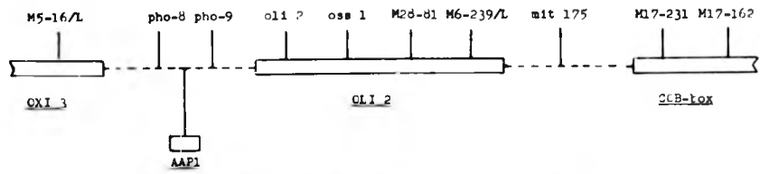

Fig. 1. The enclosed boxes represent recognised genes while the dotted lines represent intergenic regions containing hitherto unassigned mutations

sequences of mitochondrial genes. The *pho-8* and *pho-9* mutants show some potential in achieving the latter of these goals for a mitochondrial ATP synthetase gene.

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7.2 Materials and Methods

Materials

All chemicals used were of analytical grade. All restriction enzymes and DNA modifying enzymes were purchased from commercial sources (BRL, BCL). Aha III was a gift from P & S Biochemicals. Ligase was either from BRL or obtained as a gift from Anglia Biotechnology. When lyophilised restriction endonucleases were purchased (Lyphozymes, BRL), they were reconstituted according to the supplier's recommendations. Bacterial alkaline phosphatase (BAP) and calf thymus intestinal phosphatase (CIP) were purchased from Sigma. The antibiotics ampicillin, tetracyclin, and chloramphenicol were also purchased from Sigma. Cycloheximide was purchased from BDH. Electrophoretic grade agarose was purchased from Sigma or BRL. Low melting point (LMP) agarose was purchased from BRL. Acrylamide and bis-acrylamide were purchased from Sigma or BDH. Urea was purchased from Fisons or BDH. Trizma base and boric acid were purchased from Sigma. Caesium chloride was purchased from Fisons. The indicator reagents BCIG (X-gal or 5-bromo-4-chloro-3-indoyl-ß-D-galactoside) and IPTG (isopropyl-ß-D-thiogalactopyranoside) were purchased from BRL. Polyethylene glycol 6,000 was purchased from BDH. The DNA binding dyes ethidium bromide, bis-benzymide (Hoechst-33258) and DAPI (4'-6-diamidino-2-phenyl indole dihydrochloride) were purchased from Sigma. All unlabelled deoxy and dideoxy nucleotide triphosphates were purchased from BCL. ^{32}P-labelled deoxynucleotide triphosphate (α-^{32}P-dGTP and α-^{32}P-dCTP) and ^{35}S-labelled methionine were purchased from Amersham. Nitrocellulose sheets were purchased from Schleicher and Schull. X-ray film was purchased from Fuji. Finally, anti-F_1-ATPase antiserum was a kind gift from Professor G.Schatz, Biozentrum, Basel.

Stock Cultures

The yeast strains utilised in this chapter, and their growth conditions, have been detailed in Chapter 6 and again in the preceding introductory paper.

E. coli strains HB101 (Bolivar and Backman, 1979), and JM103 (Messing et al, 1981), were used for the propagation of plasmids and M13 single stranded phage respectively (see Table 7.i for genotypes).

Cloning Vehicles

The high copy number derivative of pBR322, pAT 153, was used for the cloning of mt-DNA fragments (Twigg and Sherratt, 1980). Suitably amplified mt-DNA fragments were further subcloned into the replicative forms of the M13 single stranded phage vectors mp8, mp9 (Messing and Viera, 1982; and Viera and Messing, 1982), mp10 and mp11 (Messing, 1983), for sequencing by the dideoxy chain termination method (Sanger, 1977; and Sanger et al, 1980).

Methods

1. Isolation of mt-DNA from Yeast

Mitochondrial DNA was isolated by bis-benzimide/CsCl buoyant density ultracentrifugation as essentially described by Hudspeth et al, 1980. Briefly, the yeast mitochondrial fraction was isolated as described in Chapter 6, with the exception of 250mM mannitol replacing 0.5M sorbitol in the breaking buffer. The mitochondrial pellets were lysed in lysis buffer (10mM Tris/HCl pH 7.5, 10mM EDTA, 100mM NaCl and either 2 sarkosyl or 1 SDS) and repeatedly extracted with phenol: chloroform: isoamylalcohol (24:24:1). The DNA was finally precipitated from the aqueous fraction by making the solution 0.3M Na-acetate and adding 2 volumes of ethanol at -20^{o} C. The DNA precipitate was pelleted at 4^{o}C by centrifugation at 10,000 x g and taken up in TE (10mM Tris/HCl pH 7.5, 1mM EDTA). To this, solid caesium chloride was added to give

Table 7.ia *E. coli* strain

Strain	Genotype	Remark
HB101	*F* , hsd S20 (*rB* , *mB* , rec A13, ara-14, ProA2, lacY1, galK_2, rps L20 (*Sm*^{r}), xyl-5, mtl-1, sup E44, λ	This strain is an hybrid of *E. coli* K12 and *E. coli* B. For details, see Boyer & Rouland-Dussoix (1969); Bolivar & Bockman (1979)
JM103	Δ (lac pro), thi, str A, end A, sbcB15, hsd$R^{(-)}$ 4, sup E, F traD36, proAB, lac I^q, Z ΔM15	A host of K-12 origin, restriction minus but modification plus. modification plus. See Messing *et al*, 1981 for details.

Table 7.ib M-13 vectors and their cloning sites.

Vector	Cloning site	References
mp7	EcoR1 - BamH1 - Sall/Acc1/HincII--PstI - Sall - BamH1 - EcoR1	Messing *et al* (1980)
mp8	EcoR1 - Smal - BamH1 - Sall/Acc1/HincII--Smal - HindIII	Messing & Viera (1982)
mp9	HindIII- PstI - Sall/Acc1/HincII--Smal - EcoR1	Viera & Messing (1982)
mp10	EcoR1 - SstI - Smal - BamH1 - Xbal--Sall/Acc1/HincII - PstI - HindIII	Messing (1983)
mp11	HindIII - PstI - Sall/Acc1/HincII--Xbal - BamH1 - Smal - SstI - EcoR1	"

a refractive index of 1.390 and finally, bis-benzimide added to a final concentration of 100 µg/ml. Ultracentrifugation was performed in a Beckman L5-65 machine at 45K r.p.m. (at 15°C) for 24-42 hours using the 50 Ti rotor.

The resulting DNA bands were collected under long-wave UV illumination, the mt-DNA migrating towards the low density zone or the top of the gradient. The nuclear DNA, r-DNA satellites and covalently closed 3µm plasmids banded successively at increasing densities. The resulting CsCl fractions were extracted with CsCl saturated isopropanol to remove bis-benzimide before dialysis for 24 hours at 4°C against repeated changes of TE buffer.

CsCl gradients containing DAPI and ethidium bromide were also employed, but in general proved less effective.

2. Isolation of Plasmid DNA

Plasmid DNA was isolated either in large scale or in mini scale preparations.

Large scale preparation of Plasmid

Plasmid DNAs were isolated in large scale according to the method of Katz et al, (1973). 15-20ml of LB medium containing the appropriate antibiotics (Ampicillin 100µg/ml or Tetracyclin 12.5µg/ml) was inoculated with a single bacterial colony. They were grown overnight with vigorous shaking at 37° C. Next morning, this overnight late log culture was inoculated into one litre of LB medium with the proper antibiotic (with or without 0.5 glucose) and shaken for 4-6 hours at 37° C. (The A_{600} of the culture would be approximately 0.4). Then 5ml of a solution of chloramphenicol (34mg/ml in ethanol) was added to it (final concentra- of chloramphenicol was 170 µg/ml) and incubated at 37°C with vigorous shaking for a further 12-16 hours (overnight). These cells were harvested by centrifugation at 5000 r.p.m. for 10 minutes at 4°C (Sorvall

GSA rotor) and washed with 20ml of 10mM Tris-Cl, 1mM EDTA, pH 8.0. The cells were resuspended in 8.4ml of 25% sucrose, 50mM Tris-Cl, pH 8.0 and transferred to an Erlenmeyer flask. Then 1.4ml of lysozyme (10mg/ml in 50mM Tris-Cl, pH 8.0) was added to it and kept on ice for 5 minutes with gentle swirling. 4.6ml of 0.25mM EDTA (pH 8.0) was added to it slowly and kept on ice for another 10 minutes with gentle swirling. Then 9.6ml of lysis mix (50ml 20% Triton, 125ml 0.25M EDTA, 25ml 1M Tris-Cl, pH 8.0 and 300ml water) was added to it slowly with swirling. After 10 to 15 minutes the chromosomal DNA was spun out either at 19,000 r.p.m. (Sorvall SS34 rotor) for 1 hour or at 30,000 r.p.m. (Beckman Ti 50 rotor) for 45 minutes. The supernatant was extracted with phenol two or three times and adjusted to 0.3M Na-acetate before the addition of two volumes of cold ethanol. The DNA was spun down at 10,000 r.p.m. for 10 minutes at 4^{o} C and redissolved in TE buffer (10mM Tris-Cl, pH 7.5, 1mM EDTA). About 10gm of CsCl was dissolved into the DNA solution and the final volume was made to 11ml (a refractive index of 1.30). Ethidium bromide was added to it to a final concentration of about 200 to 300 µg/ml (from a 10mg/ml stock solution). The solution was centrifuged at 45,000 r.p.m. for 36-40 hours in a Beckman 50 Ti or 65 rotor at 15^{o} C. The plasmid bands were collected either with a fine tipped Pasteur pipette, or with the help of a syringe. Ethidium bromide was extracted (three times) with isoamyl alcohol (saturated with CsCl) from the collected fractions which were then dialysed against TE buffer (two or three changes) at 4^{o} C overnight. DNA was precipitated with 2 volumes of ethanol and 0.3M Na-acetate (final concentration at -70^{o}C for 3 hours or at -20^{o}C overnight).

Mini-scale praparaion of Plasmid ("Mini-lysate" method)

This procedure was generally followed to screen a large number of transformants at a time. The method is more or less similar to that

described by Birnboim & Doly (1979). Small shaking overnight cultures (about 1ml each) were centrifuged in a 1.5ml microfuge tube (Eppendorf tube) for 2 to 3 minutes. The supernatant was discarded and the cells were resuspended in 0.1ml lysis solution (25mM Tris-Cl, pH 8.0, 10mM EDTA, 50mM or 1% glucose, 2mg/ml lysozyme) by vortexing and left on ice for 30 minutes. Then 0.2ml of alkaline SDS solution (0.2M NaOH, 1% SDS) was added and the tubes were left on ice for 5 minutes with occasional mixing by inversion. The suspensions should become viscous, then 0.15ml of high salt solution (3M Na-acetate, pH 5.0) was added, mixed and the tubes were left on ice for a further hour. A heavy white precipitate should form which was removed by centrifuging for 10 minutes at room temperature. The supernatants were transferred to new microfuge tubes. 1ml of ethanol was added to each tube and placed at -20°C for 30 minutes. They were centrifuged again in the microfuge for 3-5 minutes. The pellets were then dissolved in 0.1ml of 0.1M Na-acetate (pH 6.0) and 2.0ml of cold ethanol was added to them and left at -20°C for 10 minutes. They were centrifuged again for 5 minutes and the DNA pellets were dried under vacuum. The dried DNA pellets were dissolved in 20-50µl of TE buffer which could be digested with most restriction enzymes without further purification.

3. Isolation of ss DNA from M13

The ssDNAs were isolated using the procedure described by Sanger et al, (1980). Briefly, 1:40th dilution of a fresh overnight culture of JM103 in 2 x TY was divided into 1ml aliquots. Phage plaques were inoculated into diluted JM103 with the help of sterile toothpicks. They were incubated at 37°C with shaking for about 7 hours. The culture was poured into 1.5ml microfuge tubes and centrifuged for 10 minutes. The supernatant was transferred into another 1.5ml microfuge tube (Eppendorf) making no effort to transfer completely. The supernatant

was either stored at 4°C at this stage, or the procedure was continued to completion. In the former case the supernatant must be re-centrifuged to remove any cells which grew during storage before isolation of phage DNA. The pellets were stored at -20°C with 7.5 DMSO as a stock of the recombinant phage. To the supernatant 200 µl of PEG solution (20 polyethylene glycol (PEG), 2.5M NaCl) was added and left at room temperature for 30 minutes. The viral pellets were collected by centrifugation for 5 minutes in a microfuge. The supernatant was discarded and any trace amount of supernatant was wiped out with a Kim wipe or removed by a drawn out Pasteur pipette. To the white phage pellets 100µl of TE (10mM Tris, pH 7.5, 0.1mM EDTA) were added, and vortexed for 5 seconds to suspend the virus. The suspended virus particles were re-vortexed for 10 seconds in the presence of 50 µl of phenol and kept standing at room temperature for 10 minutes. The suspension was re-vortexed for another 10 seconds and microfuged for 3-4 minutes. The aqueous phase was collected carefully with the help of drawn out Pasteur pipettes and transferred to a fresh Eppendorf tube. The aqueous phase was extracted twice, either with 2 volumes of chloroform or 2 volumes of diethyl ether to remove PEG and traces of phenol. 10µl of 3M Na-acetate and 250µl of ethanol were added to the finally extracted aqueous solution and placed at -20° C overnight. Phage DNAs were collected by centrifugation for 10 minutes at 4°C in a Sorvall SS-34 rotor using a suitable adaptor. The DNA pellets were washed with 1ml of cold ethanol in a microfuge at room temperature, dried under vacuum and dissolved in 15 to 20µl of TE buffer (10mM Tris-Cl, pH.7.5, 10mM EDTA) and stored at -20°C.

4. Restriction Enzyme digests and Gel-electrophoresis

Restriction endonuclease digests were performed as recommended by the suppliers, before heat shocking at 65° C for 3-5 minutes with

the addition of 10% glycerol, 0.01% bromophenol blue as gel loading buffer. Agarose (1-1.5%) gel-electrophoresis was performed in open tanks as submarines in 50mM Tris-Borate, 1mM EDTA, pH 8.3 buffer. The gels were run at 40mA for 3-4 hours before staining in electrophoresis buffer plus 1 µg/ml ethidium bromide for 20 minutes. The gels were observed and photographed using a Fotodyne UV transilluminator and Polaroid CU5 land camera fitted with a Kodak 23A Wratten filter.

5. Nick Translation

^{32}P-labelled probe DNA was obtained as described by Rigby et al. (1977), using the following protocol:

5µl 10 x nick translation buffer (0.5mM Tris/HCl pH 7.2, 0.1M Mg SO_4, 1mM DTT, 1mg ml BSA).

1µg DNA

1 nmole each of cold dNTPs (minus the hot component)

100 pmoles of α-^{32}P-dNTP (25µCi)

0.01µg DNase 1

3-5 units of E.coli DNA polymerase 1

H_2O to make 50µl total volume and incubate for 1 hour at 15^{o}C.

The reaction was terminated by the addition of 5 µl 250mM EDTA pH 8.0. The unincorporated dNTPs were removed by selective isopropanol precipitation of the DNA with 2.5M NH_4 -acetate.

6. Southern Hybridisation and Dot Blot

The method of Southern, 1975, was used as described in Maniatis et al, 1982. Dot hybridisation was performed to identify recombinant M13 single stranded clones. This was performed by spotting out 2µl of single strand preparation onto nitrocellulose allowing the filter to air dry and baking for 2 hours at 80^{o}C in vacuo. The filter was then washed in 6 x SSC before prehybridisation and hybridisation, as described in Maniatis et al, 1982.

7. Colony Hybridisation

Colony hybridisation was performed using the method of Grunstein and Hogness, (1975), as described in Maniatis et al, (1982).

8. Isolation of Competent Cells

About 2ml of 2 x TY or LB media were inoculated with JM103 or HB101 respectively, from single colonies and incubated overnight at 37°C in standing culture. 10ml of the appropriate medium was inoculated by a 10 fold dilution of the overnight culture and allowed to grow at 37°C with shaking until 0.3-0.4 A_{600} was achieved, approximately 3 hours. The cells were harvested after chilling on ice at 7,000 x g for 5 minutes. The pelleted cells were resuspended in a half volume of 50mM CaCl and kept on ice for 20 minutes. The cells were centrifuged again and resuspended in a 1/10th volume of 50mM CaCl . The resulting competent cells were kept on ice for a further 40 minutes before use in transformation.

9. Ligation and Transformation

Plasmid Vector:

Ligation Mix

50-100ng dephosphorylated linearised vector DNA
>100ng target DNA with compatible ends
2µl 10 x ligase buffer (0.7M Tris/HCl pH 7.5, 70mM $MgCl_2$)
2µl 100mM ATP
2µl 50mM DTT
1 or 2 units of T4 DNA ligase
H_2O to make 20µl total volume and incubate overnight at 15°C

Add 0.2ml of competent HB101 and keep on ice for 30 minutes, heat shock at 42°C for 2 minutes, add 1ml of LB and incubate for 1 hour at 37°C before plating on LA plates plus 50µg/ml ampicillen and incubate overnight.

M13 Vector

Ligation Mix

20ng dephosphorylated linearised vector DNA
>50 ng target DNA with compatible ends
2µl 10 x ligase buffer
2µl 10mM ATP
2µl 50mM DTT
1 or 2 units of T4 DNA ligase
H_2O to make 20µl total volume and incubate overnight at 15°C

Add 0.3ml of competent JM103 and keep on ice for 30 minutes, heat shock at 42°C for 2 minutes and add to H-Top agar at 45°C containing 15µl of 2% X-gal, 25µl of 2.5% IPTG and 0.2ml of exponentially growing JM103. Plate the above mixture on minimal + glucose plates and incubate overnight.

10. Gel Purification of DNA

DNA was size fractionated by gel-electrophoresis (usually restriction fragments) and the relevant sized DNA excised from the LMP agarose gel. The gel slice was diluted by half in TE before melting at 65°C and repeated phenol extraction to produce a clear aqueous/organic interphase. The aqueous phase was then collected, extracted with diethylether and concentrated by ethanol precipitation.

11. DNA Sequencing by the Dideoxy Chain Termination Method

(Sanger, 1977; Sanger et al, 1980)

A. Primer Annealing Reaction

5µl (approximately 0.5 g) template DNA
2µl (4ng) 15bp primer oligonucleotide
1µl 10 x Klenow buffer (70mM Tris HCl, pH 7.5, 70mM $MgCl_2$, 0.5M.NaCl)
4.5µl H_2O
12.5µl Total

The above mixture was sealed in a microfuge tube and placed in an 85°-90°C water bath for 5 minutes, then the whole bath was allowed to cool slowly to room temperature.

B. Dideoxy Sequencing Reaction

The following reactions were carried out with α-^{32}P-dGTP and as such are not suitable for use with other radioactive deoxynucleotides, without further manipulation. The following solutions were prepared and stored at -20°C.

A^O Mix:

20µl	0.5mM dTTP
20µl	0.5mM dCTP
1µl	0.5mM dATP
1µl	0.05mM dGTP
20µl	10 x Klenow buffer

G^O Mix:

20µl	0.5mM dATP
20µl	0.5mM dTTP
20µl	0.5mM dCTP
1µl	0.05mM dGTP
20µl	10 x Klenow buffer

T^O Mix:

20µl	0.5mM dATP
20µl	0.5mM dCTP
1µl	0.5mM dTTP
1µl	0.05mM dGTP
20µl	10 x Klenow buffer

C^O Mix:

20µl	0.5mM dATP
20µl	0.5mM dTTP
1µl	0.5mM dCTP
1µl	0.05mM dGTP
20µl	10 x Klenow buffer

ddA	(1.0mM):	diluted 1:10
ddG	(0.7mM):	diluted 1:14.5
ddt	(2.0mM):	diluted 1:5
ddC	(0.35mM):	diluted 1:28.5

After annealing was completed, the following additions were made:

1µl (10µCi) α-^{32}P-dGTP
1µl 0.1M DTT
1µl (1 unit) large fragment DNA polymerase I (Klenow)

3µl aliquots were dispensed into microfuge tubes labelled A, G, T and C, containing 1µl each of the appropriate N^{O} and ddN solutions. The reactions were then allowed to proceed for 15 minutes at 30°C before adding the cold chase, 1µl of 0.5mM dGTP, and incubating for a further 15 minutes. The reactions were terminated by the addition of 10 µl of formamide-dye mix (0.1% (W/V) xylene cyanol, 0.1% bromophenol blue, 10mM EDTA, 95% deionised formamide) and placed on ice before boiling and loading onto the gel.

C. Gel Electrophoresis and Autoradiography

8% or 6% denaturing polyacrylamide gels containing 8M urea and 100mM Tris Borate, 10mM EDTA gel buffer were used. The gels were covered with cling film, then covered with X-ray film and frozen at -70° C overnight.

12. In Vivo Labelling of Yeast Proteins

Yeast cells were labelled with ^{35}S-methionine as spheroplasts, essentially as described by Nelson and Schatz, (1980). Briefly, yeast were grown to exponential phase in YPGal before harvesting and suspending in labelling media at 30°C for 3-4 hours. The radioactive probe was then initiated with 100µCi of ^{35}S-methionine to 100ml of the original culture volume. The cells were incubated for a further hour before chasing with 1% casaminoacids for 2 hours. The spheroplasts were then lysed and mitochondria isolated as described in Chapter 6. The labelled procedure was performed with or without the drug cycloheximide (100 µg/ml) to inhibit cytoribosome protein synthesis and specifically label mitochondrial translation products.

13. Immunoprecipitation of the OS-ATPase Complex

Labelled mitochondria were extracted with 0.5% Triton X-100 and the non-solubilized protein removed by centrifugation in a Sarstedt microfuge 3 x 10 minutes at 4°C. The clarified supernatant was then added to and equal volume of antisera prepared against purified F_1-ATPase and incubated with gentle shaking overnight at 4°C. The immunoprecipitate was centrifuged out at 4°C in the Sarstedt microfuge for 10 minutes and washed twice with 25mM phosphate buffer pH 7.5, 0.5% Triton X 100.

14. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed as 20cm x 20cm x 1mm slab gels as essentially described by Lamelli, (1970). Routinely, gradient gels were utilised, composed of 8.5-12.5% and 10-15% gradient slabs with 6.25% and 8.5% stacking gels, respectively. The immunoprecipitates were suspended in 40µl of sample buffer, boiled and loaded onto the gels which were run overnight at 15-20mA. The resulting radioactive bands were fixed in 10% methanol, 10% acetic acid and 80% water for 1 hour and visualised by fluorography as described by Bonner and Laskey, (1974).

7.3 Results and Discussion

The Mitochondrial ATPase Complex

In parallel to DNA sequencing studies, the mitochondrial translation products and mitochondrial ATPase components were examined for abnormalities by *in vivo* labelling in the presence of cycloheximide and immunoprecipitation of holo-mtATPase from a Triton extract by anti F_1-ATPase. Figure 7.ii demonstrates the immunoprecipitable bands of the ATPase complex. The bands visualised here are those which remain associated with the complex after mild detergent extraction, as the antisera used will only recognise the F_1 portion of the enzyme complex, and therefore may not be completely representative of the full functional entity. Bearing this limitation in mind, the following analysis may be made.

Figure 7.iic and d show clearly the immunoprecipitable mitochondrially translated Fo components of the OS-ATPase complex, the ubiquitous protein labelled subunits 6, 8 and 9 corresponding to the known genes Oli 2, AAP1 and Oli 1 respectively (see Roberts *et al*, 1979, and Marazuki *et al*, 1983). The Oli 2 gene product appears normal from the mutant strain CD40 lending more evidence that drug resistant mutations are point mutations not disturbing the integrity or apparent function of the ATPase complex. The holo-mtATPase complex (Figure 7.iib) appears to consist of 10 subunits, the apparent molecular weights of which are inset. The visible F_1 components of which have been identified by running simultaneously on the same gel purified F_1-ATPase and staining separately with coomassie blue. This would appear to leave 2 subunits unaccounted for, which are apparently nuclear encoded and synthesised on cytoribosomes. Candidates for these are the OSCP and the possible yeast equivalents of F_6 and Factor B, although their molecular masses are at variance.

Figure 7.ii

The Immunoprecipitable Bands of the OS-ATPase Complex

A. Immunoprecipitate of holo-ATPase labelled in the absence of cycloheximide (-CHX)

B. Purified F_1 ATPase

C&D. The immunoprecipitable bands of halo-ATPase labelled in the presence of cycloheximide (+CHX)

Figure 7.ii

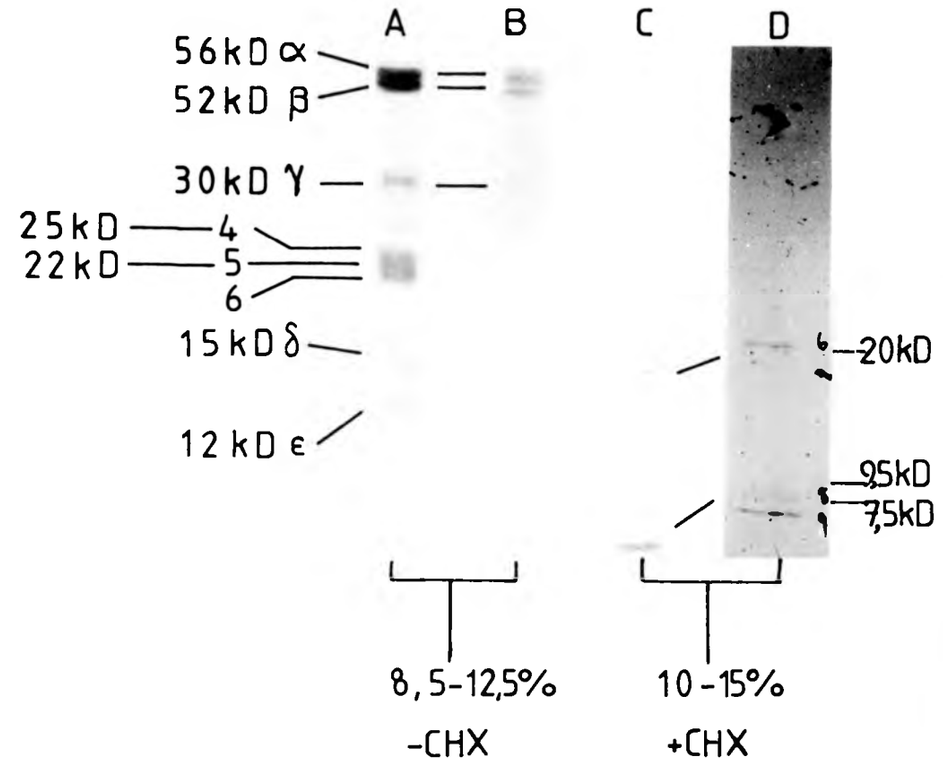


Figure 7.ii

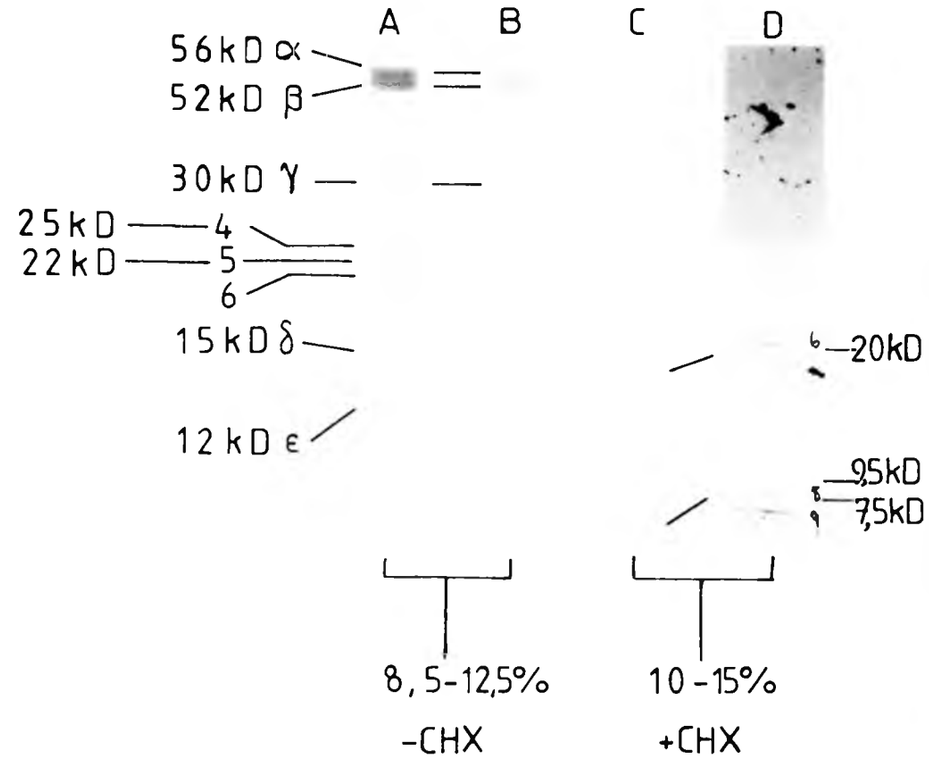
The Immunoprecipitable Bands of the OS-ATPase Complex

A. Immunoprecipitate of holo-ATPase labelled in the absence of cycloheximide (-CHX)

B. Purified F_1 ATPase

C&D. The immunoprecipitable bands of halo-ATPase labelled in the presence of cycloheximide (+CHX)

Figure 7.ii



Cloning of the Oli 2 Region

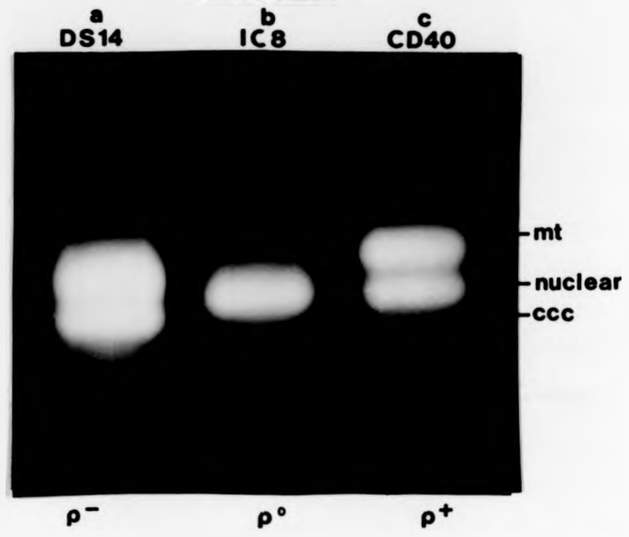
The initial strategy employed was to clone the Eco-6 and Eco-7 fragments of the grande mitochondrial genomes in the plasmid vector pAT-153. These fragments were known to contain the Oli 2 gene, ascertained by comparing the genetic and physical maps of grandes and petites rescuing the loci responsible for oligomycin resistance (Prunell et al, 1977; Sanders et al, 1977; and Morimoto and Rabinowitz, 1979a and b). The pedigrees of all the strains employed here were based on the mitochondrial genome of D273/10B, the so-called short mitochondrial genome omitting some of the introns of the mosaic genes Oxi 3 (the gene coding for subunit 1 of cytochrome oxidase) and Cob (the gene coding for apocytochrome b), diagnosed by producing nine EcoR1 fragments after digestion and resolution by agarose gel electrophoresis. Clones of the Eco-6 and Eco-7 fragments were obtained by 'shotgunning' EcoR1 generated restriction fragments of the grande mitochondrial genome into EcoR1 linearised vector pAT153. These clones were identified by colony hybridisation using a ^{32}P-labelled probe, obtained from the petite genome DS-14 containing the Oli 2 gene (Macino and Tzagoloff, 1980). The positively identified recombinants were then screened for the nature of their inserts by EcoR1 digestion of mini-prep lysates. Such preparations yielded either 1.7Kb Eco-7 or 2.55Kb Eco-6 bands on agarose gel electrophoresis, the correct clones were named pSCXME6 and 7 where X delineates the parental yeast strain. Figure 7.iii shows a flow diagram of the experiments involved in this procedure.

Initial restriction maps were obtained for these clones based on single, double restriction enzyme digests and end labelling of the excised gel fragments and partial digestion with restriction enzymes (Smith and Birnstiel, 1976). Restriction maps of these fragments are presented in Figure 7.iv.

Table 7.ii Recombinant *E. coli* clones and the parental yeast strains.

Name of the recombinants	Parental yeast strain	Mitochondrial genotype
pSC273ME6 & pSC273ME7	D273-10B/A1	ρ^+ *Oli2*^{S} *Oss1*^{S}
pSC27ME6 & pSC27ME7	D27	ρ^+ *Oli2*^{S} *Oss1*^{S}
pSC603ME6 & pSC603ME7	D603-3B	ρ^+
pSC22ME6 & pSC22ME7	D22	ρ^+
pSC40ME6 & pSC40ME7	CD40	ρ^+ *Oli2*^{R} *Oss1*^{R}
pSC24ME6 & pSC24ME7	CD24	*mit* pho9
pSC41ME7	CD41	*mit* pho8
pSC175ME6 & pSC175ME7	mit-175	*mit*
pSC76ME6 & pSC76ME7	D27/76	ρ^+ *Oli2*^{R} *Oss1*^{S}
pSC92ME7	D27/92	ρ^+ *Oli2*^{S} *Oss1*^{R}
pSC14ME6 & pSC14ME7	DS14	ρ^- *Oli2*^{R}

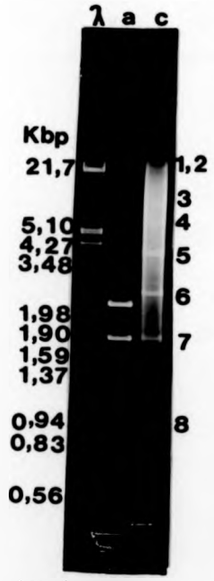
Fig.7.iii



CsCl / Bis-benzimide gradient

↓

EcoR1 digestion of mt DNA

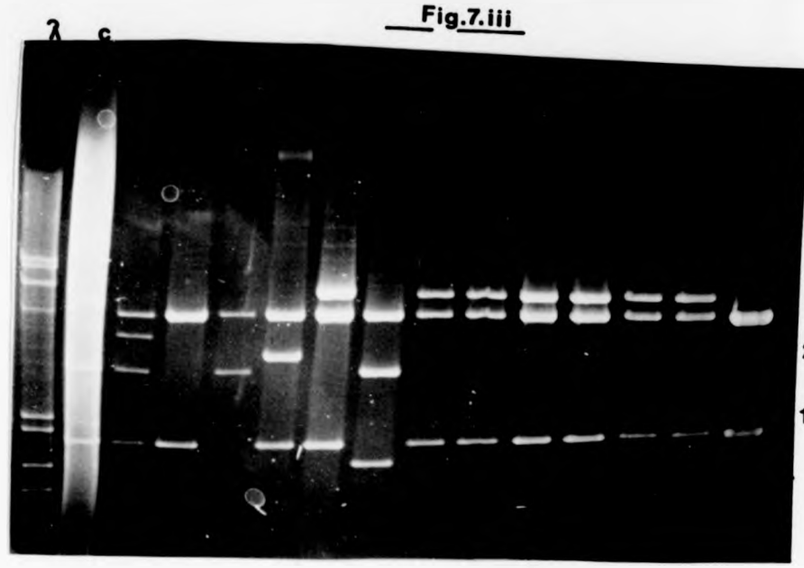


Ligate into pAT 153

→

Patch out Amp^R colonies

Fig.7.iii



'mini-prep' plasmid

EcoR1 digest

Colony hybridise

→

^{32}P-labelled DS14 mtDNA

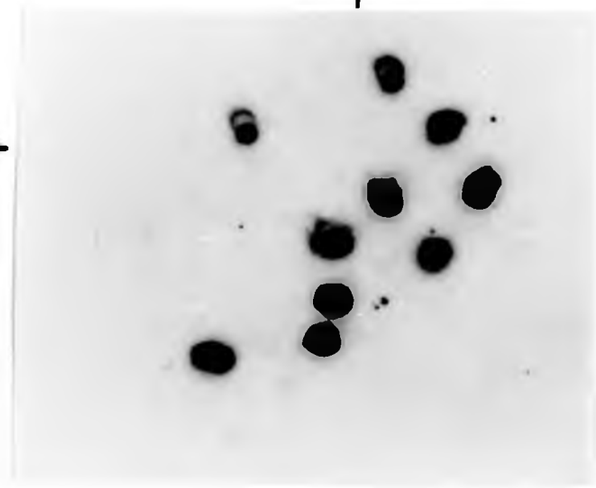
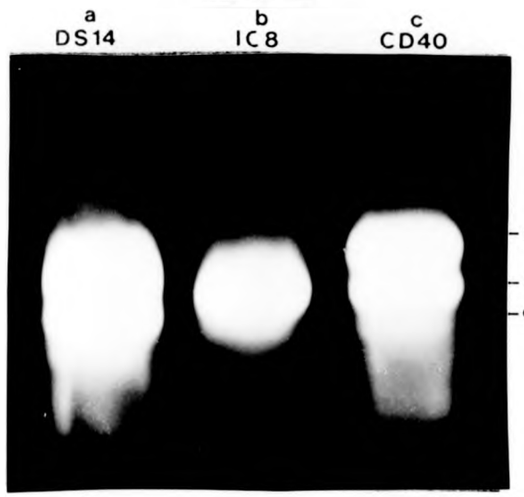


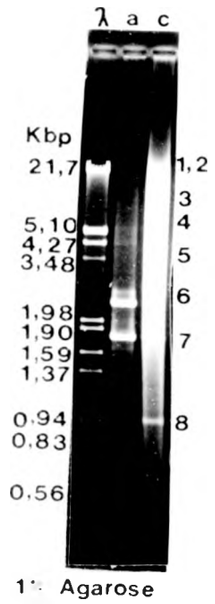
Fig.7.iii



CsCl / Bis-benzimide gradient

↓

EcoR1 digestion of mt DNA



Ligate into pAT 153

→

Patch out Amp^R colonies

'mini-prep' plasmid

EcoR1 digest

Fig.7.iii

Colony hybridise

→

^{32}P-labelled DS14 mtDNA

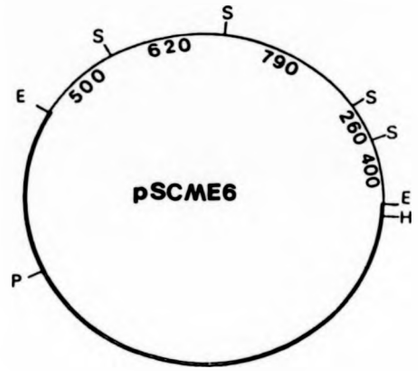
Figure 7.iv

Restriction Maps of pSCME6 and pSCME7 Clones

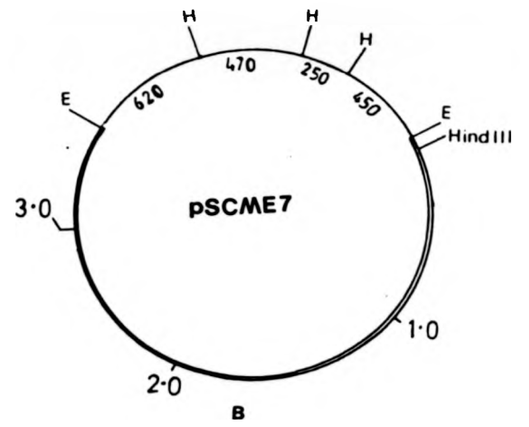
Legend:

A. Restriction map of pSC4OME6 showing the ordered Sau 3A fragments and their respective approximate sizes in base pairs of the inserted mitochondrial DNA.

B. Restriction map of pSC4OME7 showing the ordered Hpa II fragments and their respective approximate sizes in base pairs of the inserted mitochondrial DNA.



A



B

E = EcoR1

H = Hpa II

S = Sau 3A

P = Pst 1

Nucleotide Sequence of the Oli 2 Region

The complete nucleotide sequence of the Oli 2 region is presented in Figure 7.v from the grande strain CD40 which bears the resistance alleles for *Oli*^{R}2-76 and *Oss*^{R}1-92. The sequence spans 4,349 bp comprised of two *EcoR1* restriction fragments cloned originally in pAT-153, the first stretching from the C-terminal end of the Oxi 3 gene to the C-terminal end of the Oli 2 gene, including the Aap1 gene sequence, some 2,571 bp. The second *EcoR1* fragment is continuous with the first about the *EcoR1* site within the Oli 2 gene coding sequence and extends 1,784 bp downstream including a long putative open reading frame interrupted by a series of 'G+C' rich sequences (RF3). The subcloning and sequencing strategy employed in this study is shown in Figure 7.vi superimposed on the above reading frames. The codon usages of these genes is presented in Table 7.iii, where, as expected, the third base bias is towards A or T.

The AAP1 Gene

The Aap1 gene (ATPase associated protein or subunit 8 of OS-ATPase, Macreadie *et al*, 1983), starts at 1,017th nucleotide and spans a reading frame of 144 bp corresponding to a protein product of 48 aminoacids and 5.5 K Daltons molecular weight. The reading frame commences with *f-met* (ATG) and is terminated by an ochre codon (TAA) which is again repeated in frame 27 bp downstream. The ochre codon has been used for all yeast mitochondrial genes examined so far. 199 bp upstream of the ATG initiation codon is the modified consensus sequence (-ATATAAGTA-) which is the putative transcriptional initiation sequence (Tabak *et al*, 1983). However, evidence has been produced based on 'Northern' blot, S1-mapping and *in vitro* capping experiments that the Oli 2 region is co-transcribed with the mosaic Oxi 3 gene (Hensgens *et al*, 1983; Christianson and Rabinowitz, 1983; Tabak *et al*, 1983;

Figure 7.v

Strain	Genotype
CD40	a leu hsr 1-1,2* 01[illegible] 2-76 Oss* 1-92
0273-108/A1	a met 2* 01[illegible] Oss*


CD40 AGAAATAATAAAAAATATTAATAAGAATATAATTTAATAATTATTAAAAAAATTCTTATTTATAGTCCGGTCCGCCCCCGCGGGGCGGACCCCAAAGGAGGAGTAATAAAAATTATTAAA
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
MspAI
CD40 TACAAATATTATATATATATAATTCATTATATATATATATATATAATAATTAATCTTATTTTTATATATTTATTTATATATCTATTTATATTTATATTTTATATATATTTATTATATA
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
CD40 TCTAAGGGGTTTGGTCCCTCCCCCGTAAGTATAAGTATACGGGGGAGGGTCCCTCACTATTTATATTTATATTTATATTTATATTTATATTTATATTTTATATATTTTATATATTTATAA
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
Oli2
MetPheAsnLeuLeuAsnThrTyrIleThrSerProLeuAspGlnPheGlu
CD40 ATAAAGTATAATAAGATATAATTATGATTAATTATTTATAAGTTATAGTTTTATAAATTTATAATTATTATGTTTAATTTATTAAATACATATATTACATCACCATTAGATCAATTTGAG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
AhaIII Sau3AI
IleArgThrLeuPheGlyLeuGlnSerSerPheIleAspLeuSerCysLeuAsnLeuThrThrPheSerLeuTyrThrIleIleValLeuLeuValIleThrSerLeuTyrLeuLeuThr
CD40 ATTAGACTATTATTTGGTTTACAATCATCATTTATTGATTTAAGTTGTTTAAATTTAACAACATTTTCATTATATACTATTATTGTATTATTAGTTATTACAAGTTTATATCTATTAACT
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
AhaIIIAhaIII
AsnAsnAsnAsnLysIleIleGLyserArgTrpLeuIleSerGlnGluAlaIleTyrAspThrIleMetAsnMetThrLysGlyGlnIleGlyGlyLysAsnTrpGlyLeuTyrPhePro
CD40 AATAATAATAATAAAATTATTGGTTCAAGATGATTAATTTCACAAGAAGCTATTTATGATACTATTATAAATATGCTTAAAGGACAAATTGGAGGTAAAAATTGAGGTTTATATTTCCCT
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
MetIlePheThrLeuPheMetPheIlePheIleAlaAsnLeuIleSerMetIleProTyrSerPheAlaLeuSerAlaHisLeuValPheIleIleSerLeuSerIleValIleTrpLeu
CD40 ATGATCTTTACATTATTTATGTTTATTTTTATTGCTAATTTAATTAGTATGATTCCATATTCATTTGCATTATCAGCTCATTTAGTATTTATTATCTCTTTAAGTATTGTTATTTGATTA
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280
Sau3AI HinfI
GlyAsnThrIleLeuGlyLeuTyrLysHisGlyTrpValPhePheSerLeuPheValProAlaGlyThrProLeuProLeuValProLeuLeuValIlePheGluThrLeuSerTyrIle
CD40 GGTAATACTATTTTAGGTTTATATAAACATGGTTGAGTATTCTTCTCATTATTCGTACCTGCTGGTACACCATTACCATTAGTACCTTTATTAGTTATTTTTGAAACTTTATCTTATATT
D273/10BA1 ATT
2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
AlaArgAlaIleSerLeuGlyLeuArgLeuGLyserAsnIleLeuAlaGlyHisLeuLeuMetValIleLeuAlaGlyLeuLeuPheAsnPheMetLeuIleAsnLeuPheThrLeuVal
CD40 GCTAGAGCTATTTCATTAGGTTTAAGATTAGGTTCTAATATCTTAGCTGGTCATTTATTAATGGTTATTTTAGCTGGTTTACTATTTAATTTTATGTTAATTAATTTATTTACTTTAGTA
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520
PheGlyPheValProLeuAlaMetIleLeuAlaIleMetIleLeuGluPheAlaIleGlyIleIleGlnSerTyrValTrpThrIleLeuThrAlaSerTyrLeuLysAsnThrLeuTyr
CD40 TTCGGTTTTGTACCTTTAGCTATGATTTTAGCTATTATGATTTTAGAATTCGCTATTGGTATTATCCAATCTTATGTTTGACTTATCTTAACAGCATCATACTTAAAAAATACATTATAC
D273/10BA1 GAT
2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640
EcoRI
LeuHis***
MetLysLysGlnAsnLeuAsnSerIleLeuLeuMet
CD40 TTACATTAAATTATAAAATAAAAATTATATAAAAAAAAATATAATAATAATAATATAAAATAAAAAAATAAAAAATAAAACAATGAAAAAACAAAATTTAAATTCTATTTTATTAA
2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760
TyrIleAsnTyrIleIleAsnTyrPheAsnAsnIleHisLysAsnGlnLysLysAspTrpIleMetGlyTyrGluTyrMetTyrLysPheLeuMetAsnAsnMetThrCysPheIleLysTrp
CD40 TGTATATTAATTATATTATTAATTATTTTAATAATATTCATAAAATCAATTAAAAAGACTGAATTATAGGATATGAATATATATATAAATTTTTAATAAATAATATACTATGTTTTATTAAATGA
2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880


AspAsnAsnLysIleLeuLeuLeuLeuAspMetTyrTyrAsnValLeuTyrAsnTyrHisLysGlnArgThrProMetSerAsnLysArgLeuMetAsnSerLysAsnIleMetValPro
***PheArg
CD40 GATAATAATAAAATTTTATTATTATTAGATATATATTATAATGTATTATATAACTATCATAAATAAGTATATCTATATCTAATAAAAGATTAATAAATTTAAAAAATATTATAGTTCCG
2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
GlyProGlyHisGlySerArgAsnProAlaArgArgLeu***
GlyProAlaThrGlyAlaGlyThrProGlnGlyAspTyrLysLeuLeuTyrThrTyrPheTyrIleLeuAsnLysMetLysMetGluMetAspAsnTyrAsnAsnAsnAsnAsnIleSer
CD40 GGGCCCGGCCACGGGAGCCGGAACCCCGCAAGGAGATTATAAATTATTATATCTTTATTTTTATATTTTAAATAAAATAAAAATAGAAATAGATAATTATAATAATAATAATATTT
3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
LeuLysTyrAsnGluLeuLeuLysAsnIleMetAsnAsnLeuAsnTyrLysLeuSerAsnIleGluThrAsnLeuSerAsnAsnPheTyrLeuMetAspLysTyrLeuIleAsnLysTyr
CD40 CATTAAAAATATAATGAATTATTAAAAAAATATTATAAATAATTTAAATTATAAACTATCTAATATTGAACTTAATTTATCTAATAATTTTTATTTAATAGATAAATATTTAATTAATAAATAT
3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240
***SerArgProGlnProGlyProArgLysGluAsnMetLeuAsnMetIleProAsnAsnTyrMetPheAsnAsnIleAsnTyrLysGlyLysLeuAsnIleLysThrValLeu
MetLysTyrLeuValProAlaProAlaGlyThrProGlnGlyGluTyrIleLysTyrAsnSer***
CD40 ATAAAATATTTAGTCCCGGCCCCAGCCGGGACCCCGCAAGGAGAATATATTAAATATAATTCCTAATAATTATATATTTAATAATATTAATTATAAAGGTAAATTAAATATTAAAACAGTATTA
3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350
---------- P1 ----------
AspLeuAsnAsnAsnGluPheTyrAspTyrLeuSerGlyLeuIleGluGlyAspGlyTyrIleGlyProLysGlyIleThrIleThrAsnHisAlaAsnAspValLeuAsnThrIlePheIle
CD40 GATTTAAATAATAATGAATTTTATGATTATTTATCAGGGTTAATTGAAGGTGATGGTTATATTGGTCCTAAAGGTATTACAATTCTAAATCATGCTAATGATGTATTAAATACTATCTTTATT
3360 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480
AsnLysArgIleLysAsnSerIleLeuValGluLysTrpMetAspThrLeuLysAspAsnProTyrPheValAsnAlaPheSerIleAsnIleLysThrAsnLeuAlaLysGluLysIle
CD40 AATAAAAGAATTAAAAATAGTATTTTAGTAGAAAAATGAATAGATACTTTAAAAGATAATCCTTATTTTGTTAATGCTTTCTCTATTAATATTAAACTTAATTTAGCTAAAGAAAAGATT
3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
PheThrAsnIleTyrAsnLysLeuTyrSerAspTyrLysIleAsnGlnIleAsnAsnHisIleProTyrTyrAsnTyrLeuLysIleAsnAsnLysLeuProIleLysAsnIleMetValPro
***PheArg
CD40 TTTCTTAATATTTATAATAAATTATATAGTGATTATAAAATTAATCAAATTAATAATCATATTCCTTATTATAATTATTTAAAAATTAATAATAAATTACCTATTAAAAATATTATAGTTCCG
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720
GlyProGlyHisGlySerArgAsnProThrArgArgTyr*** ----------P2----------
GlyProAlaThrGlyAlaGlyThrProGlnGlyAspIleLysAsnAsnTyrTrpLeuAlaGlyPheThrAlaAlaAspGlySerPheLeuSerSerMetTyrAsnProLysAspThrLeuLeu
CD40 GGGCCCGGCCACGGGAGCCGGAACCCCACAAGGAGATATTAAAAATAATTATTGATTAGCTGGTTTTACAGCTGCAGATGGTTCTTTTTTATCATCTATATATAATCTAAAGATACATTATTA
3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830
PheLysAspMetArgProSerTyrValIleSerGlnValGluThrArgLysGluLeuIleTyrLeuIleGlnGluSerPheAspLeuSerIleSerAsnValLysLysValGlyAsnArgLys
CD40 TTTAAAGATATAAGACCTAGTTATGTTATTTCACAAGTTGAAACACGTAAAGAATTAATCTATTTAATTCAAGAATCTTTTGATTTATCTATTTCTAATGTTAAAAAAGTTGGTAATAGAAAA
3840 3850 3860 3870 3880 3890 3900 3910 3920 3930 3940 3950
LeuLysAspPheLysLeuPheThrArgThrThrAspGluLeuMetLysPheIleTyrTyrPheAspLysPheLeuProLeuHisAspAsnLysGlnPheAsnTyrIleLysPheArgPheAsn
CD40 TTAAAAGATTTTAAATTATTTACCAGAACTCTTGATGAATTAATAAAATTTATTTATTATTTTTGATAAATTTTTTACCTTTACATGATAATAAACAATTTAATTATATTAAATTTAGATTTAAT
3960 3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080
LeuPheIleLysSerTyrAsnTrpAsnAsnArgValPheGlyLeuValLeuSerGluTyrIleAsnAsnIleLysIleAspAsnTyrAspTyrTyrTyrTyrAsnLysTyrIleAsnMet
CD40 CTATTTATTAAATCATATAATTGAAATAATAGAGTCTTTGGTTTAGTATTATCTGAATATATCAATAATATTAAAATTGATAATTATGATTATTATTATTATAATAAATATATTAATATA
4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
HisAsnAlaArgLysProLysGlyTyrIleLys***
CD40 CATAATGCACGTAAACCTAAAGGATACATTAAATAATTAATTATCCTTACTATTATAATTATTCTATATATTATATATAAAAATAAATATATAAAATTTTATAATACAAAAAGAATTC
4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320
EcoRI

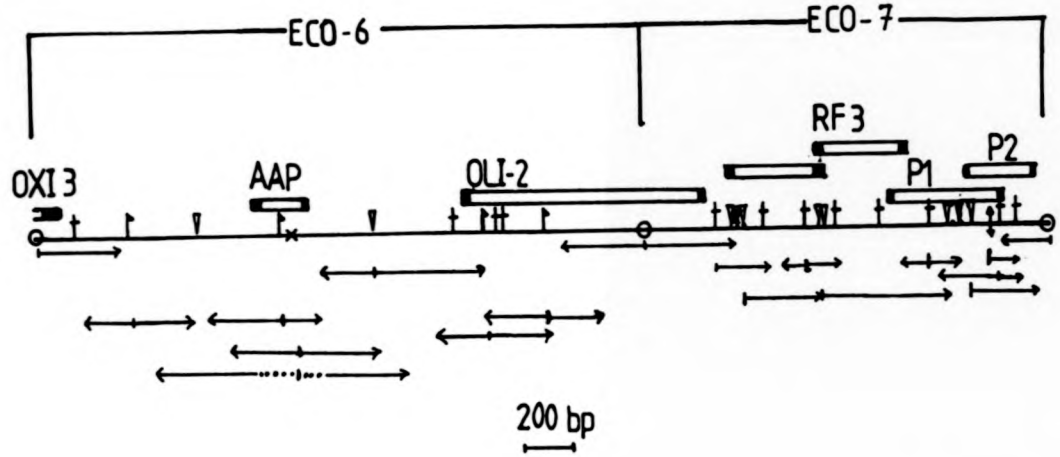


Figure 7.vi

Sequencing Strategy Employed for the Sequencing of the Oli 2 Region

o = EcoR1

↕ = Pst 1

ᚴ = Sau 3A

X = Xba I

▽ = Hpa II

† = Aha III

Table 7.iii Codon Use by the mitochondrial genes for the O.S.ATPase

The data for subunit-9 has been taken from Macino & Tzagoloff (1979).

Aminoacid	Codon	Subunit-6	Subunit-8	Subunit-9
Ala	GCA	2	0	4
	GCT	11	0	5
	GCC	0	0	1
	GCG	0	0	0
Arg	AGA	4	2	1
	AGG	0	0	0
	CGA	0	0	0
	CGT	0	0	0
	CGC	0	0	0
	CGG	0	0	0
Asp	GAT	4	0	0
	GAC	0	0	1
Asn	AAT	14	1	1
	AAC	0	0	1
Cys	TGT	1	0	1
	TGC	0	0	0
Glu	GAA	3	0	1
	GAG	1	0	0
Gln	CAA	5	3	1
	CAG	0	0	0
Gly	GGA	2	0	2
	GGT	14	1	8
	GGC	0	0	0
	GGG	0	0	0
His	CAT	4	0	0
	CAC	0	0	0
Ile	ATT	31	3	7
	ATC	5	1	2
Leu	TTA	47	12	11
	TTG	0	0	0
Lys	AAA	5	1	2
	AAG	0	0	0
Met	ATG	9	4	3
	ATA	1	0	0
Phe	TTT	15	4	1

codon.use (Contd.)

	TTC	6	3	6
Pro	CCA	4	2	1
	CCT	4	1	1
	CCC	0	0	0
	CCG	0	0	0
Ser	TCA	11	1	5
	TCT	5	2	0
	TCC	0	0	0
	TCG	0	0	0
	AGT	3	0	0
	AGC	0	0	0
Thr	ACA	9	1	2
	ACT	6	0	0
	ACC	0	0	0
	ACG	0	0	0
	CTA	3	1	1
	CTT	2	0	0
	CTC	0	0	0
	CTG	0	0	0
Trp	TGG	0	0	0
	TGA	5	0	0
Tyr	TAT	9	3	1
	TAC	2	0	0
Val	GTA	7	1	5
	GTT	5	1	1
	GTC	0	0	0
	GTG	0	0	0
TOTAL		259	48	76

Osinga et al, 1984; and Simon and Faye, 1984). It is quite possible in vivo that mRNA synthesis may originate as a long co-transcript of Oxi 3, Aapl, Oli 2 and RF3, but additionally a shorter co-transcript may also occur from upstream of Aapl, certainly such a sized RNA has been identified on 'Northern' blots (Ray, 1985).

The predicted molecular weight of this protein is 5.5 K Daltons, which is not observed on immunoprecipitates of OS-ATPase. Certain Aapl mit^- mutants have been correlated with the loss or aberrant mobility of a 10 K Dalton protein on SDS-PAGE, the apparent gene product (Macreadie et al, 1983; and Marazuki et al, 1983). These observations have been rationalised with the purification and aminoacid sequence determination of the protein (Velours et al, 1984). The aminoacid sequence confirms the DNA sequence predicted molecular weight of the protein, and it has further been observed that the purified protein migrates according to the predicted molecular weight on formamide gel systems. It remains to be discovered whether the observed 10 K Dalton protein is a functional dimer as is the hexameric structure predicted for subunit 9 of OS-ATPase (or the DCCD binding protein, Tzagoloff et al, 1976; and Tzagoloff and Akai, 1972).

The predicted structure of subunit 8 is a single transmembrane α-helix with charged groups extending either side of the lipid bilayer. Other homologous poteins have been discovered within various mitochondrial genomes (see Figure 7.viii) although E.coli has no apparent homologous counterpart. However, the predicted secondary structure of the E.coli subunit b protein closely resembles the subunit 8 predicted structures and have been proposed to be analogous (Ray, 1985).

The Oli 2 Gene

The Oli 2 gene (or subunit 6 of OS-ATPase, Roberts et al, 1979), is located 709 bp downstream of the Aapl gene and constitutes a 777 bp

open reading frame of 259 codons. The predicted molecular weight of the polypeptide product is 28.5 K Daltons, which is somewhat higher than the observed polypeptide product of some 20 K Daltons on SDS-PAGE (see an earlier section of this chapter; Roberts et al, 1979; Marazuki et al, 1983; Somlo et al, 1982 and 1985). The predicted aminoacid sequence of this protein is very hydrophobic and can potentially form seven transmembrane α-helices (see Ray, 1985 for a detailed study). The primary aminoacid sequences and predicted secondary structures are similar to those predicted from DNA sequences of other mitochondrial genomes (see Figure 7.vii and Figure 7.viii).

The drug resistant mutations were located in CD40 by comparison with wild type sequences at the 171st and 254th residues for oligomycin and ossamycin. These changes have been confirmed by sequencing the singly resistant strains D27/76 and D27/92 (see Figure 7.ix and 7.x respectively). Oligomycin resistance appears to be associated with a single T→A transition, corresponding to the replacement of a highly conserved isoleucine residue with phenylalanine. An exchange in this codon has also been observed by Macino and Tzagoloff, (1980), associated with oligomycin resistance of the allele Oli^R 2-118 where the same isoleucine residue had been substituted with methionine. A third Oli 2 allele has been investigated by Novitski et al,(1984); the Oli^R2-23 allele is associated with the exchange of a conserved serine residue in the 175th residue to threonine. This region of the subunit 6 gene (the 'Oli 2 block') appears to be conserved throughout evolution, and in addition to binding oligomycin may be functionally involved in the energy conservation system of the OS-ATPase.

Another oligomycin resistant mutation has been located towards COOH-terminus of the subunit 6 gene, the Oli^R4-622 allele defining a second recombination group within the gene or the 'Oli 4 block' (Macino and

Figure **7.vii** Comparison of aminoacid sequences of the subunit-6 of the O.S.ATPase from various organisms. (1) E. coli, (2) Aspergillus nidulans (3) Saccharomyces cerevisiae, (4) Bovine, (5) Human (6) Mouse, (7) Drosophila melanogaster, (8) Drosophila yakuba, The aminoacid sequences have been arranged to establish maximum homology.
A "-" indicates nothing but a gap within the sequences to establish homology.

```
1.MASENMT----PQDYIGHHLNNLQLDLRTFSLVDPQNPQNPPATFWTINIDSMFFSVVLGLLFLVLFRSVAKK
2.MYQ--FNFILSPLDQFEIRDLFSLNANVLGNIHLSITNIGLYLSIG-------------LLL-TLGYHL-AHN
3.MFNLLNTYIFSPLDQFEIRTLFGLQSSFIDLSCLNLTTFSLYIIV--------------LLVITSLYTLTNNN
4.MN---------------EN--LFT---SFITPVILGLPLVTL--IVLF-PS---------LLFPTSNR-LV--S
5.MN---------------EN--LFT---SFIAPTILGLPAAVL--ILLF-PP---------LLIPTSKY-LI--N
6.MN---------------EN--LFA---SFITPTMMGFPIVVA--IIMF-PS---------ILFPSSKR-LI--N
7.MMTNLFSVF----DP-----LAIFNFSLNWLSTFLGLLMIPS-IYWLMPSRYNIMWNS-ILLTLHK-------
8.MMTNLFSVF----DP-----LAIFNLSLNWLSTFLGLLMIPS-IYWLMPSRYNIMWNS-ILLTLHK-------

ATSGVPGKFGTAIELVIGFVNGSVKDMYHGKSKLIAPLALTIFVWVFL------------MMNLMDLLPYIAEHV
NKIPNN-WSISQEAIYATVHSIVINQLNPTKGQL------------YFP-FIYALFIFILVNNLIGMVPYSFAST
NKIIGSRWLISQEAIYDTIMNM--------KGQIGG----KNWWGLYFP-MIFTLFMFIFIANLISMIPYSFALS
NRFVTLQWMLQLVS----------------K-QMMSIHNSKGQYWTL---MLMSLILFIGSTNLLGLLPHSFTPT
NRLITTQWLIKLTS----------------K-QMMTMHNTKGRTWSL---MLVSLIIFIATTNLLGLLPHSFTPT
NRLITTQWLIKLTS----------------K-QMMLIHTPKGRTWTL---MIVSLIMFIGSTNLLGLLPHSFTPT
------------EF----------------KTLLGPSGHHNGSTFIFISL--FSLILFNNFM---GLFPYIFTST
------------EF----------------KTLLGPSGHHNGSTFIFISL--FSLILFNNFM---GLFPYIFTRT

LGLPALRVVPSADVNVTLSMALFIGVLILFYSIKMKGIGFTKELTLGPFNHWAFIPVNLILEGVSLL----SKPV
SHFILTFSMSFTIVLGATF-LGLQRHGL-------K----FFSLFVPSGCPLGLLPLLVLIEFISYL----SRNV
AHL------VFIISLSIVIWLGNTILGLY------KHGWVFFSLFVPAGTPLPLVPLLVIIETLSYI----ARAI
TQL------SMNLGMAIPLWAGAVITGF------RNKTKASLAHFLPQGTPTPLIPMLVIIETISLFIQPMALA-
TQL------SMNLAMAIPLWAGTVIMGF------RSKIKNALAHFLPQGTPTPLIPMLVIIETISLLIQPMALA-
TQL------SMNLSMAIPLWAGAVITGF------RNKLKSSLAHFLPQGTPISLIPMLIIIETISLFIQPMALA-
SHLTLT------LSLALPLWLCFMLYGW------INHTQHMFAHLVPQGTPAILMPFMVCIETISNIIRPGTLA-
SHLTLT------LSLALPLWLCFMLYGW------INHTQHMFAHLVPQGTPAILMPFMVCIETIRNIIRPGTLA-

SLGLRLFGNMYAGELIFILIAGLLPWWSQWILNVPWAIFIFHILIITL-----------QAFIFMVLTIVYLSMA
SLGLRLAANILSGHMLLSILSGFTYNIMTSGILFFFLGLIPLAFIIA-FSGLELAIAFIQAQVFVVLTCSYI---
SLGLRLGSNILAGHLLMVILAGLTFNFMLNLFTLVFGFFVPLAMILA-IMILEFAIGIIQSYVWTILTASYL---
---VRLTANITAGHLLI-HLIGGATLALMSISTTTALITFT---ILILLTILEFAVAMIQAYVFTLLVSLYL---
---VRLTANITAGHLLM-HLIGSATLAMSTINLPSTLIIFT---ILILLTILEIAVALIQAYVFTLLVSLYL---
---VRLTANITAGHLLM-HLIGGATLVLMNISPPTATITFT---ILLLLTILEFAVALIQAVVFTLLVSLYL---
---VRLTANMIAGHLLLTLL-GNTGSS-MSYM----LMTFLLMAQIALLV-LESAVAMIQSYVFAVLSTLYS---
---VRLTANMIAGHLLLTLL-GNTGPS-MSY-----LLVFLLVAQIALLV-LESAVTMIQSYVFAVLRTLYS---

SE---E-H
KD-GLDLH
KD-TLYLH
HDNT
HDNT
HDNT
SEVN
REVN
```

Figure 7.viii Comparison of Amino acid Sequences of Subunit-8 from Various Organisms.

* indicates same aminoacid in more than two phylogenetic groups. Organisms are: 1 Aspergillus amstelodemi, 2 Aspergillus nidulans, 3 Saccharomyces cerevisiae 4 Human, 5 Bovine, 6 Mouse, 7 Drosophila melanogaster 8 Drosophila yakuba. "-" has been introduced within the sequences to establish maximum homology. Homologous regions have been shown in colour. Pink, common to all or fungi only; yellow, mammals and insects or mammals only orange, insects only.

```
  ****                                  *       *             *
1.MPQLVPFFFVNQVV-YAFVNL-TVLIYAFTK---FIIPKLLRIFISRIVI----------NKL
2.MPQLVPFFFVNQVI-FAFIVL-TVLIYAFTK---YILPRLLRTYISRIYI----------NKL
3.MPQLVPFYFMNQLITYGFLLM-ITLLILFSQ---FLLPMILRLYVSRLFI----------SKL
4.MPQLNTTVWPTMITPMLLTL-FLITQLKMLNTNYHLPPSP-KPMKMKNYNK---PWEPKWTKICSLHSLPPQS
5.MPQLDTSTWLTMILSMFLTL-FLITQLKVSKHNFYHNPELTPTKMLKQNT----PWETKWTKIYLPLLLPL
6.MPQLDTSTWFITISSM-ITLFFLLTQLKVSSQTFPLAPSPKSLTTMKVKT----PWELKWTKIYLPHSLPQQ
7.IPQLAPISWLLLFIIFSTL--FLLTCSINYYSYMPNSPKSNEL---KNINLNSMNW--KW
8.IPQLAPIRWLLLFIVFSTL--FLLTCSINYYSYMPTSPKSNEL---KNINLNSMNW--KW
```

Figure 7.ix

Nucleotide Changes at the Oli2 Locus

Legend:

A. Autoradiogram of a sequencing gel of the recombinant clone 84066 containing the Oli^R 2-76 allele at the nucleotide A marked with an arrow. The wild type sequence indicates a T at the corresponding position resulting in a aminoacid change of methionine to phenylalanine.

B. The similar nucleotide change found in the singular resistant strain D27/76 confirming the nucleotide change responsible for oligomycin resistance.

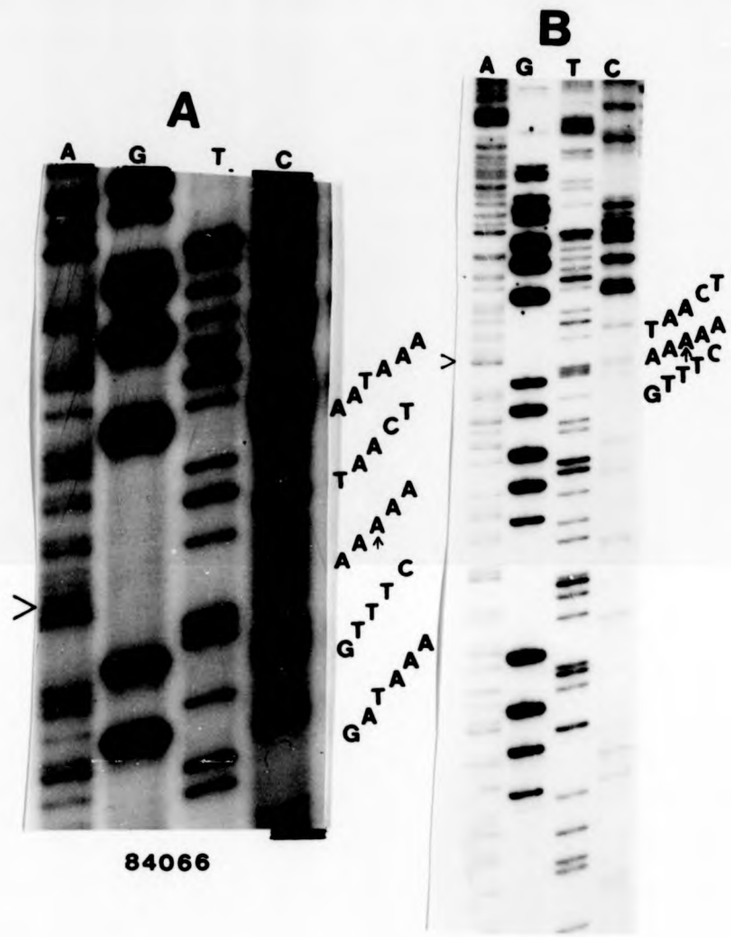
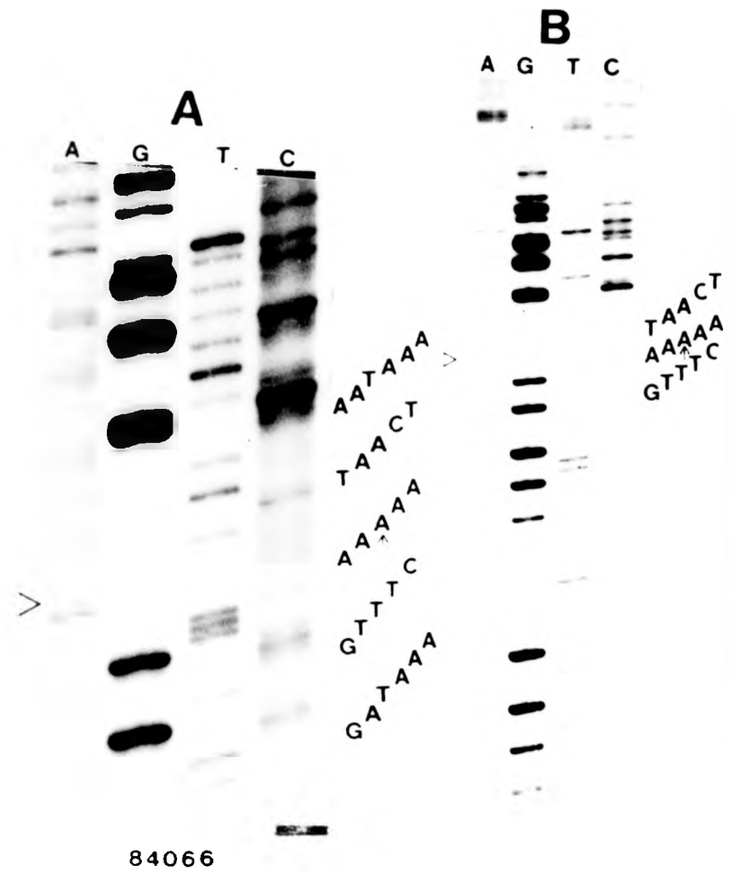


Figure 7.ix

Figure 7.ix

Nucleotide Changes at the Oli2 Locus



Legend:

A. Autoradiogram of a sequencing gel of the recombinant clone 84066 containing the Oli^R 2-76 allele at the nucleotide A marked with an arrow. The wild type sequence indicates a T at the corresponding position resulting in a aminoacid change of methionine to phenylalanine.

B. The similar nucleotide change found in the singular resistant strain D27/76 confirming the nucleotide change responsible for oligomycin resistance.

Figure 7.ix

Tzagoloff, 1980). The mutation is due to the alteration of the 232nd residue, where a conserved leucine is changed to phenylalanine. Also within the 'Oli 4 block' Slott et al, (1983) have identified a codon change responsible for oligomycin resistance in a mouse cell line where a conserved valine has been replaced by a glutamic acid residue.

Both the Oli 2 and Oli 4 associated changes have one characteristic in common, they occur adjacent to a glutamic acid residue within a hydrophobic segment of the subunit 6 protein. Because the mutations reported here and previously have apparently no bioenergetic handicap (see Chapter 6 and Griffiths and Houghton, 1974), they are probably not directly involved in proton translocation, but apparently interfere with the binding of oligomycin by hydrophobic interaction adjacent to charged residues where the drug may exert its effect.

Ossamycin resistivity exerted by the allele $Oss^{R}1\text{-}92$ is due to a change of a conserved aspartic acid residue to asparagine by virtue of a G→A transition in the nucleotide sequence (see Figure 7.x). The amino-acid change due to ossamycin resistance is in the 254th residue near the carboxyl terminus. This region probably lies outside the membrane as predicted from its charged nature. Therefore, it is likely that ossamycin binds by a charge interaction and causes inhibition at the initial site of transfer of the proton channel by direct interaction or conformational change.

However, ossamycin resistance may have other contributing factors in subunit 6, since certain Oli 2 mutations show cross-resistance (Lancashire and Mattoon, 1979; and Griffiths and Houghton, 1974). Therefore, other residues may have indirect effects. For example, the allele $Oli^{R}2\text{-}118$ sequenced by Macino and Tzagoloff, (1980), is cross resistant to ossamycin despite being within the same codon distinguished from oligomycin in this study. This phenomenon has been put

Figure 7.x

Nucleotide Changes at the Ossl Locus

Legend:

A. The autoradiogram shows a DNA sequencing gel of recombinant clone 82737 containing the wild type sensitive allele at the Ossl locus. The G marked with an arrow undergoes a change in the Oss^R1-92 allele.

B. The autoradiogram shows a DNA sequencing gel of recombinant clone 84077 containing the ossamycin resistant allele Oss^R1-92. The sequence is that of the non-transcribing strand and shows the G-A nucleotide transition from that of the wild type in track A.

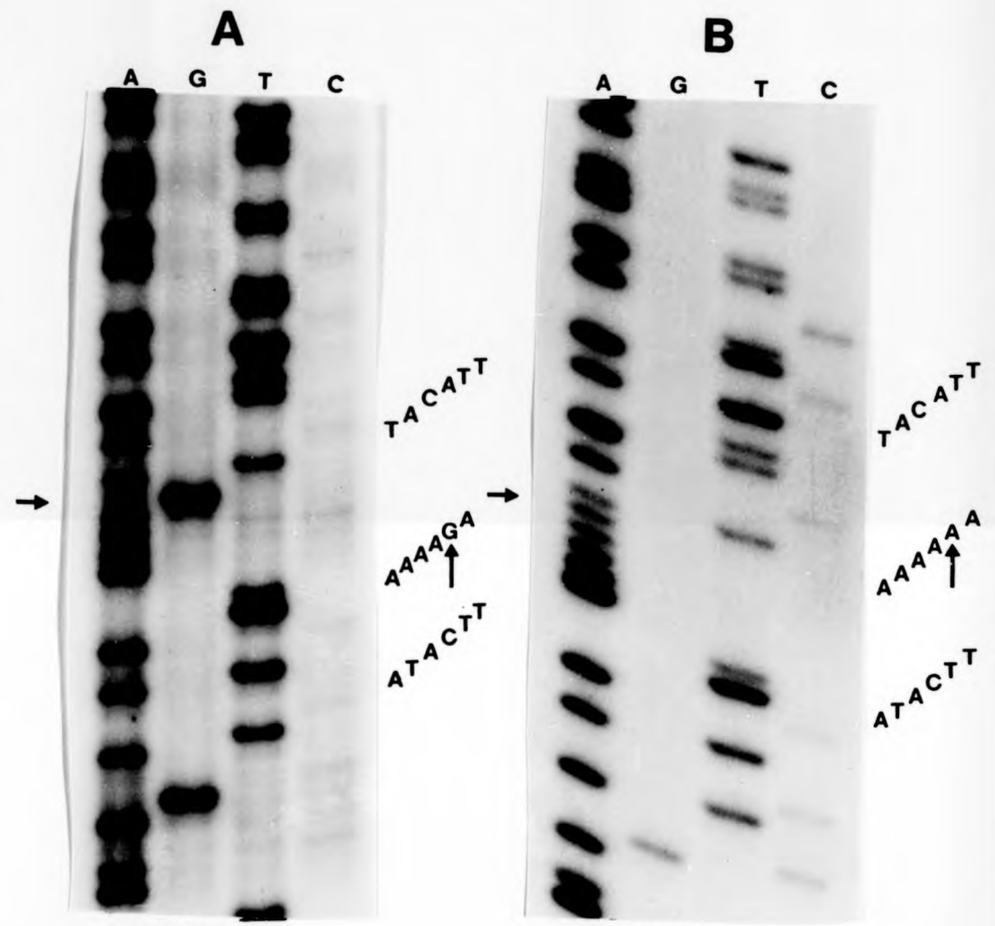


Figure 7.x

Figure 7.x

Nucleotide Changes at the Ossl Locus

Legend:

A. The autoradiogram shows a DNA sequencing gel of recombinant clone 82737 containing the wild type sensitive allele at the Ossl locus. The G marked with an arrow undergoes a change in the Oss^R1-92 allele.

B. The autoradiogram shows a DNA sequencing gel of recombinant clone 84077 containing the ossamycin resistant allele Oss^R1-92. The sequence is that of the non-transcribing strand and shows the G-A nucleotide transition from that of the wild type in track A.

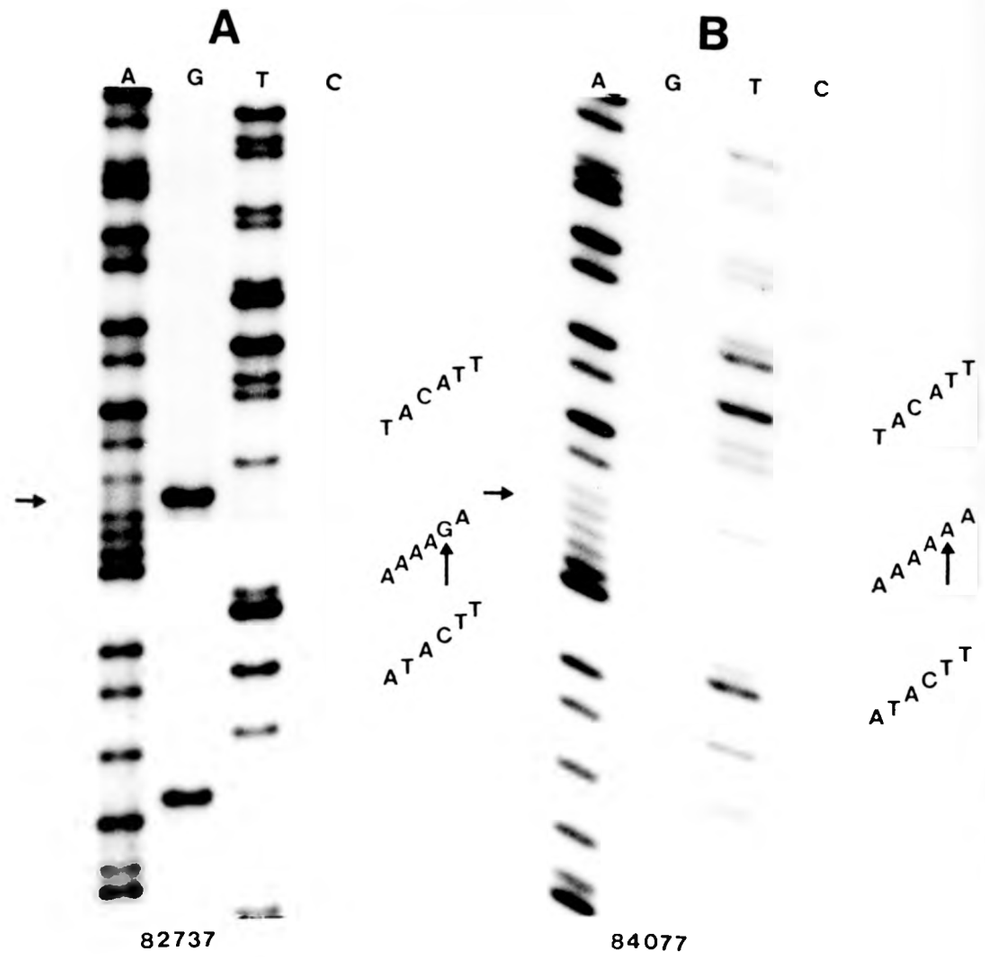


Figure 7.x

to use to examine intra-codon cross-over in the mitochondrial genome, allowing the experimenter to follow the transmission of the parental alleles, while searching for true intra-codon recombinants by their predicted sensitivities to the drugs and thereby generating further point mutations within the Oli 2 locus. Such recombinant strains have been isolated and subsequent molecular analysis is presently under investigation (Connerton et al, 1984b).

The sites of drug resistance discussed above are shown in Figure xi.

RF3

The open reading frame downstream of Oli 2 starts with an AUG codon 80 bp downstream of the Oli 2 gene and terminates at a G+C rich cluster at the 3,008th nucleotide with an opal codon. A second reading frame overlaps at +1 bp and continues until an ochre codon at the 3,323rd nucleotide just beyond a second G+C rich cluster. A third reading frame overlaps at +1 bp and terminates at nucleotide 3,780 with an ochre termination codon. Finally, a fourth ORF overlapping by +1 bp terminates at an ochre codon at nucleotide 4,266. It has been postulated that since the whole of this region is transcribed, the overlapping reading frames may be translated as a continuous polypeptide by virtue of frameshifting events forced upon the mitoribosome by the strong secondary structures imposed upon the mRNA by the internal G+C rich clusters (Seraphin et al, 1985). Such an event would give rise to a 500 aminoacid long protein, presumably at low levels, a fact which may be required physiologically. Certainly instances of frameshifting in yeast mitochondrial DNA are known, (Hensgens et al, 1984). Indeed the mitochondrial translation machinery has been shown to suppress frameshift mutations (Fox and Weiss-Brummer, 1980; and Somlo et al, 1985), even in the same transcript. Further evidence as to the expression of the putative protein comes from an examination of the aminoacid

Figure 7.xi Comparison of the drug resistant sites of the subunit-6 from various organisms. * indicates the altered aminoacid in drug resistant strain. Aminoacids are shown as single letter code.

	* *	* *
S. cerevisiae	P L L V L I E F I S Y L	L E F A I G I I Q S Y V W
A. nidulans	P L L V I I E F I S Y L	L E L A I A F I Q A Q V F
Bovine	P M L V I I E T I S L F	L E F A V A M I Q A Y V F
Human	P M L V I I E T I S L L	L E I A V A L I Q A Y V F
Mouse	P M L V I I E T I S L F	L E F A V A L I Q A V V F
Rat	P M L I I I E T I S L F	L E F V V A L I Q A Y V F
D. melanogaster	P F M V C I E T I S N I	L E S A V A M I Q S Y V F
D. yakuba	P F M V C I E T I R N I	L E S A V T M I Q S Y V F
	(a) OLI2 Mutational site	(b) OLI4 Mutational site

	*
S. cerevisiae	Y L - - - K D T L Y L H
A. nidulans	Y I - - - K D G L D L H
Bovine	Y L - - - H D N T
Human	Y L - - - H D N T
Mouse	Y L - - - H D N T
Rat	Y L - - - H D N T
D. melanogaster	Y S - - - S E V N
D. yakuba	Y S - - - S E V N
E. coli	Y L S M A S E - - E H

(c) Ossl Mutational site

sequence, which reveals two peptide motifs, P1 and P2, which are common to all maturase enzymes discovered in mitochondria (Michel et al, 1982 and Waring et al, 1982), (see Figure 7.xii). It may well be that RF3 is responsible for processing the Oxi 3-Oli 2 multigenic transcript. However, certain strains of S.cerevisiae do not possess the RF3 sequence (Cobon et al, 1982; and Seraphin et al, 1985). It may be that these strains have a completely functional protein encoded elsewhere within their mitochondrial genomes. This is possible since Michel (1984), has discovered a similar organised sequence, RF2, downstream of the Oxi 2 gene. The RF2 reading frame contains 'G+C' rich clusters found here, these sequences also occur throughout the genome of S.cerevisiae, their function as yet remains obscure (Figure 7.xiii).

A mit mutation has been localised downstream of Oli 2 (see introductory paper to this chapter), based on petite deletion mapping and its rescue by the defined petite CDS14, a cytoductant of DS14 (Macino and Tzagoloff, 1980). If this mutation resides within the RF3 sequence, then it may be translated. It has been shown that the mutation causes a loss of subunit 1 of cytochrome oxidase and the production of a series of aberrant peptide products on analysis of the mitochondrial translation products of the mutant (J. Velours, personal communication). By analogy to RNA splicing mutants of apocytochrome b (Kreike et al, 1979), the mit175 mutation may be deficient in the splicing of the Oxi 3 gene, in which case RF3 is a bona fide maturase.

Sequencing studies on the mutant strain are presently under way and have revealed only one base change so far at nucleotide 4,152 where a C→A transition is present, and results in the loss of a Hinf 1 restriction site as compared with the wild type. This base transition results in a conservative codon change, maintaining phenylalanine at the 462nd aminoacid residue. This sort of event cannot be the mutational site,

Figure 7.xii

Comparison of Peptide Motifs in Maturases

		P1		P2
Sc	RF3	-YLSGLIEGDGYI-	104aa	-WLAGFTAADGSF-
Sc	AI3	-YLAGLIEGDGSI-	120aa	-WLAILTDADGNF-
Sc	AI4	-WLAGLIDGDGYF-	94aa	-WFVGFFDADGTI-
Sc	AI5β	-YLSGLFEGDGNI-	97aa	-WLTGFNDADGYF-
Sc	BI4	-WLAGLIDGDGYF-	94aa	-WFMGFFDADGTI-
Sc	RF1	-WLVGFTDGDGSF-	124aa	-WILGFIEAEGTF-
Sc	RF2	-WLSGFVVGDGYF-	121aa	-FILGFIEAEGTF-
An	AI2	-YLAGLIEGDGTI-	107aa	-WLSGFIEADGSF-
An	AI3	-YLAGLIDGDGHF-	99aa	-WLAGFSDADASG-
An	BI1	-YLVGLFEGDGYF-	120aa	-WLVGFIEAEGCF-
Nc	BI2	-FLAGLVDGDGYI-	119aa	-WIVGFIYSKGSF-
Nc	BI2	-FLAGLVDGDGYI-	144aa	-WIVGFTCSEGSF-
Nc	OI2	-YITGFVDGEG -	158aa	-WIRGFIEGEG -
Sp	AI2	-YLAGLIDGDGHF-	99aa	-WLAGFSDADASF-
CONSENSUS		-YLAGL*DGDG+F-		-WL*GF/DADGSF-
		W S E I		I E E T

Legend:
Comparison of the dodecapeptide sequences P1 and P2 which feature in the intron ORF's of group 1B and are present in RF1 and RF2. Distances between P1 and P2 are in aminoacids. The meaning of the symbols drawn in the consensus sequence are the following: *: majority of apolar AA; +: majority of non-ionic polar AA; /: any AA. Sc: Saccharomyces cerevisiae; An: Aspergillus nidulans; Nc: Neurospora crassa; Sp: Schizosaccharomyces pombe.

Figure 7.xiii

Comparison of GC Clusters in Yeast Mitochondrial DNA

Legend:

Homologies between GC clusters, GC rich sequences related to RF3 clusters 1 or III and II are aligned to illustrate their sequence similarity. Bases diverging from the consensus are in lower case letters. Extra bases are indicated below the sequences.

The sources of the sequences are as follows:

a. Coruzzi et al, 1981.
b. Michel, 1984.
c. Hensgens et al, 1983.
d. Goursot et al, 1982.
e. Bonitz and Tzagoloff, 1980.
f. Miller et al, 1983.
g. Sor and Fukuhara, 1980.
h. Tzagoloff et al, 1980.
i. Nobrega and Tzagoloff, 1980.

Figure 7.xiii

CLASS A:

RF3 I	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGCAAGGAG	+	
RF3 II	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCaCAAGGAG	+	
RF1	TAGTTCCGGGGCCCGGCCACGGGA-CCGGAACCCCGAAAGGAG	-	(a)
RF2 I	TAGTTCtGGGGCCCGGCCACGGGAGCCGGAACCCCGTAAGGAG	+	(b)
AI5β	TAGTTtCGGGGgCCGGaCcaGGGA-CCGGAACCCCaAAAGGAG	-	(c)
ORIs1	TAGTTCCGGGGCCCGGCtACGGGAGCCGGAACCCCGAAAGGAG	-	(d)
ORIs3	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGAAAGGAG	-	(d)
ORIs4	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGGAAGGAG	+	(d)
ORIs5	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGAAAGGAG	-	(d)
ORIs6a	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGAAAGGAG	+	(d)
ORIs6b	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGAAAGGAG	+	(d)
tRNAser2	TAGTTCCGGGGCCCGGCCACGGaAGCCGG-ACCCCGAAAGGAa	+	(e)
tRNAasn	TAGTTCCGGaGCCCGGCCACGGGAGCCGGAACCCCG-AAGGAa	-	(a)
TSL	TAGTTCCGGGttCCGGCCACGGGAGCCGGAACCCCtAAAGGAG	-	(f)
15SrRNA	TAGTTCCGGGGCCCGGCCAC-GGAGCC-GAA-CCCGAAAGGAG	+	(g)
OLI1 E	TAGTTCCGGGGttCGGCCACGGGAGCCGGAACCCCGAAAGGAG	+	(h)
OLI1 B	TAGT-CCGGGGCCCGGCCAC-GGA-CCGGAACCCCGAAAGGAG	+	(h)
3'COB A	TAGTTCCGGGGCCCGGtCACGGaAGCCGGAACCCCGCAAGGAG	+	(i)
3'COB B	TAGTTCCGGGcCCCGGCCACGGGAaCCGGAACCCCcGAAGGAG	-	(i)
CONSENSUS	TAGTTCCGGGGCCCGGCCACGGGAGCCGGAACCCCGNAAGGAG		

CLASS B:

CONSENSUS	TAGTTCCCGCTTCGCGGGAACCCCGNAAGGAG		
RF2 II	TAGTTCCCGCTTCGCGGGAACCCCGTAAGGAG	-	(b)
RF2 III	cAGTTCCCGCTTCGCGGGAACCCCGTAAGGAG	-	(b)
RF2 IV	TAGTTCCCGCTTCGCGGGAACCCCGTtAGGAG	-	(b)
RF3 II	TAGTcCCgGCccCGCcGGgACCCCGCAAGGAG	+	

but merely a conservative polymorphism. This event has been observed before in the subunit 6 gene, leading to an new Sau 3A site at the 226th codon in the strains D22 and JM6 (Ray, 1985, and Novitski et al, 1984). However, such a conservative change would suggest some constraint on the system to maintain the RF3 sequence intact.

7.4 Conclusion

The nucleotide changes associated with the resistance alleles $Oli^{R}2\text{-}76$ and $Oss^{R}1\text{-}92$ have been determined. These changes occur close to conserved glutamic acid residues within the structural gene for subunit 6 of the mitochondrial ATPase complex in the yeast Saccharomyces cerevisiae. These regions may define in part the binding sites for the drugs and their inhibitory sites in blocking oxidative phosphorylation. Although these sites may overlap on subunit 6, as ascertained by the cross resistance of the $Oli^{R}2\text{-}118$ allele. The predicted aminoacid sequence of the subunit 8 gene is colinear with the experimentally determined aminoacid sequence (Velours et al, 1984).

The DNA sequence presented here also defines a new putative gene downstream of Oli 2. The sequence consists of four overlapping reading frames interrupted by G+C rich clusters. The expression of the full-length predicted polypeptide would be dependent on frameshifting events during mitochondrial translation. The putative polypeptide product would contain two peptide motifs which are indicative of a maturase activity, possibly to splice the Oxi 3 - Oli 2 multigenic transcript. The mit^{-} mutation mit175 maps in this region and affects the production of a full length functional Oxi 3 gene product, despite its apparent map position.

APPENDIX 1

Introduction

Organotin compounds have been widely used as biocidal agents (particularly triorganotins), and plastic stabilisers. The introduction of these compounds into the environment has raised concerns as to how they may interact with biogeochemical cycles. Consequently, a need has arisen for simple analytical methods for the determination of various organotin compounds within environmental and biological samples. To this end the following method was developed.

In previous studies gas chromatographic methods have been utilised for the simultaneous determination of tetraalkyl- and trialkyltins (Arakawa et al, 1981a and 1981b) which could be applied to biological extracts. However, these methods suffered from difficult preparation and failed to reproducibly detect dialkyltin species because of their adsorption and decomposition during chromatography.

Previous studies have also utilised spectrofluorometric techniques for the determination of organotins. These studies have been based on the enhanced fluorescence of 3-hydroxyflavone and 2', 3', 4', 5', 7-pentanydroxyflavone (the latter is more commonly known as morin).

The fluorescence of 3-hydroxylflavone with triphenyltin has been used to measure submicrogram amounts of this species in potatoes (Vernon, 1974), and water (Bluden and Chapman, 1978). This assay has since been further refined and used to measure triphenyltin in a variety of samples (Baker et al, 1980; Aldridge and Street, 1981). However, this approach is limited since only the trimethyl- and triphenyltin complexes exhibit fluorescent properties in aqueous solution (Blunden and Chapman, 1978; Aldridge and Street, 1981) and the presence of chloride destabilises the complex when exposed to light (Aldridge and Cremer, 1957). More recently, the fluorescent properties of

3-hydroxyflavone and organotins have been studied further, where it appears that other n-alkyltin species will form fluorescent complexes when obtained by boiling in toluene (Blunden and Smith, 1982). However, the applicability of this technique to biological materials is undoubtedly questionable.

More success, however, has been achieved using morin, which produces fluorescent complexes with mono di- and trialkyltins which produce various intensities of green fluorescence which may be calibrated with known quantities of the individual species (Arakawa et al, 1983). These complexes appear stable under laboratory conditions, and the presence of various other organometal compounds did not interfere with the assay. One drawback of the assay is the need to extract the organotin from samples into a hexane solution. Although the authors of this work presented apparently reproducible procedures for such extractions, they are time consuming. Nevertheless, the coupling of this technique with high performance liquid chromatography and thin-layer chromatography has allowed the simultaneous determination of a variety of organotin compounds (Yu and Arakawa, 1983).

During the progress of these studies, the above morin based method was reported, although it appears a sensitive and reproducible assay, it does suffer in its requirement for hexane extraction, whereas the following method is applicable in aqueous solution for n-alkyltins and, as such, may offer certain advantages to the investigator.

Materials and Methods

Reagents

Trimethyltin chloride (Me_3SnCl), triethyltin chloride (Et_3SnCl), tripropyltin chloride (Pr_3SnCl), tributyltin chloride (Bu_3SnCl) and triphenyltin chloride (Ph_3SnCl) were all purchased from BDH Chemicals Ltd. U.K. Trioctyltin chloride (Oc_3SnCl) and tricyclohexyltin hydroxide

(Oc_3SnCl) and tricyclohexyltin hydroxide (Cy_3SnOH) were purchased from Dow Chemical Company USA. Monoethyltin trichloride ($EtSnCl_3$), and diethyltin dichloride (Et_2SnCl_2) were purchased from the Ventron Corporation USA. Tributyltin oxide ($(Bu_3Sn)_2O$), tributyltin phosphate ($(Bu_3Sn)_2PO_4$), and tributyltin malate ($Bu_3SnCl_2CC_2C_2H_2CO_2CH_3$) were gifts from Schering Industrie-Chemikalien, West Germany. These organotins were made up in 2ml batches of 01.M in ethanolic solution and kept dark. 1.aniline-8-napthalene sulphonate (ANS) was purchased from Eastman-Kodak Ltd., USA. ANS was kept as a 10mM aqueous solution in the dark.

Apparatus

All fluorescence measurements were performed with a Perkin-Elmer MPF-3 spectrofluorometer in four sided 4ml quartz cuvettes. Spectrophotometry was performed on a Shimadzu R500 instrument.

General Procedure

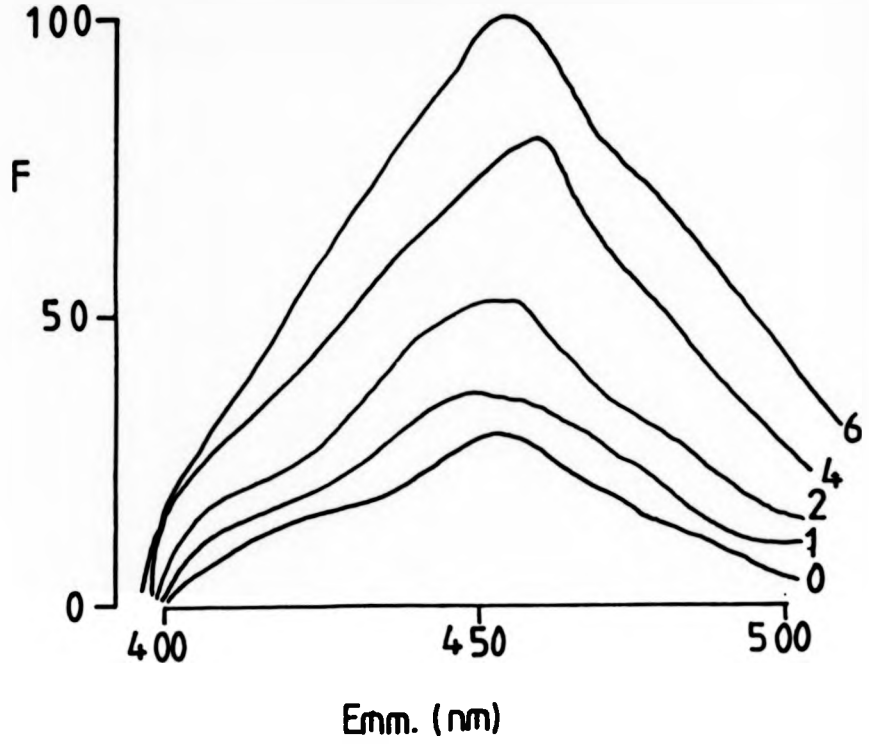
To a 4ml cuvette up to 0.1ml additions of organotin in ethanolic solution were made, before the addition of ANS and water to make 10 μM. The cuvette was mixed and immediately placed in the fluorometer (Excitation 380nm - Emission 460nm) which had been blanked with an equal concentration of ANS solution, prior to use.

Results

Principle

ANS fluoresces at 460nm when excited at 380nm, the intensity of this fluorescence may be increased by the addition of trialkyltin. The observed increase in intensity can be correlated proportionately to the addition of increased amounts of the trialkyltin species. The emission spectra presented in Figure 1Ai demonstrate this function for n-tributyltin chloride on ANS fluorescence which, in common with other trialkyltins, has no fluorescent properties of its own.

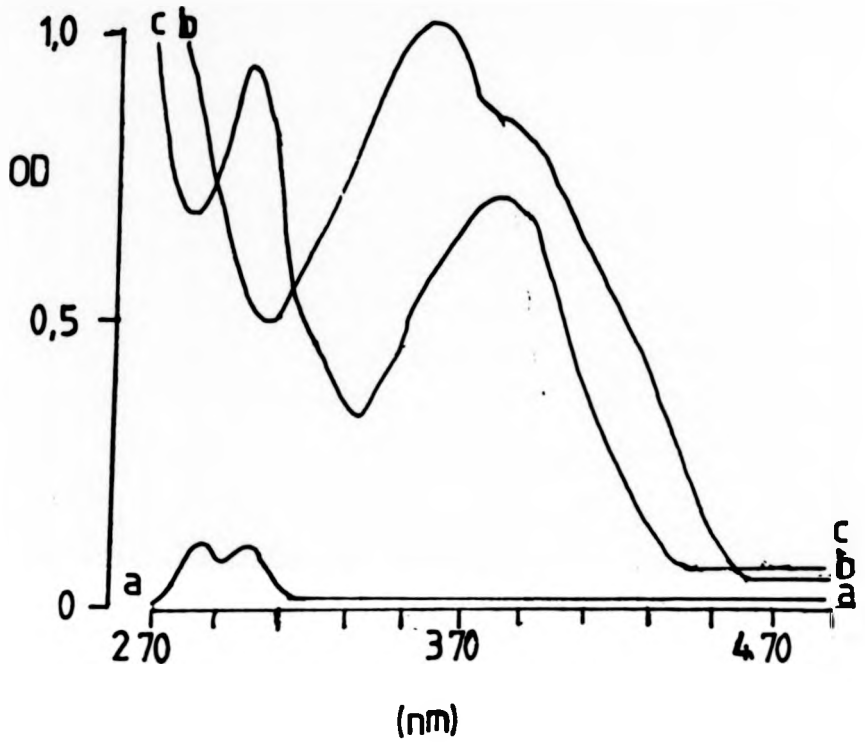
Figure 1A.i



Emmission Spectra of Tributyltin - ANS Complex

Legend:
Emission spectra of tributyltin - ANS complex excited at 380nm with 0, 1, 2, 4, and 6 x 10^{-6} M concentrations of tributyltin chloride in the presence of 25 x 10^{-6}M ANS.

Figure 1A.ii



Spectral Changes Accompanying the Addition of Equimolar Quantities of ANS and Tributyltin Chloride

Legend:

a. 0.1×10^{-3}M tributyltin chloride

b. 0.1×10^{-3}M ANS

c. 0.1×10^{-3}M ANS and tributyltin chloride

The increase in fluorescent intensity of the tributyltin -ANS complex is accompanied by a change in the absorption spectrum with the modification of peaks at 350 and 375nm and the production of a new peak at 282nm over the basic ANS spectrum. These spectral changes are presented in Figure 1Aii, which are indicative of the formation of a charge transfer complex between ANS and the trialkyltin.

Further investigation of this interaction was carried out utilising ^1H NMR (90MHz). Presented in Figure 1Aiii are a series of spectra of 10mg/ml solutions of ANS and tributyltin chloride in dutero-methanol. Tributyltin chloride produces a ^1H spectrum with a complex splitting pattern, due to the interaction of $-CH_2-$ protons with the various tin nuclei which possess spin. The alkyl protons resonate upfield (0.89 - 1.83 δ/ppm), which produce a triplet of nine equivalent CH_3 protons (0.89, 0.97 and 1,04 δ/ppm) and 3 x 6 ^1H of three non-equivalent $-CH_2-$ protons (1.16 - 1.83 δ/ppm).

ANS produces a typical downfield ^1H spectrum consisting of ten aromatic and two amino protons (6.77 - 8.42 δ/ppm).

On mixing these reagents, no change in chemical shift or splitting of the n-butyl protons were observed, however, the aromatic protons, although similarly not experiencing any appreciable change in chemical shift, do exhibit a variation in splitting, suggesting a possible close interaction with the n-butyl protons present. The exact nature of this interaction cannot be determined from this data, but changes in the $\pi \rightarrow \pi^*$ transitions experienced by the aromatic systems may be envisaged, based on the above evidence, which in turn, may certainly modify the fluorescent properties of the system.

Calibration and Detection Limits

Calibration curves were obtained using a standard 25 µM solution of ANS and making small ethanolic additions of triorganotins serially

Figure 1A.iii

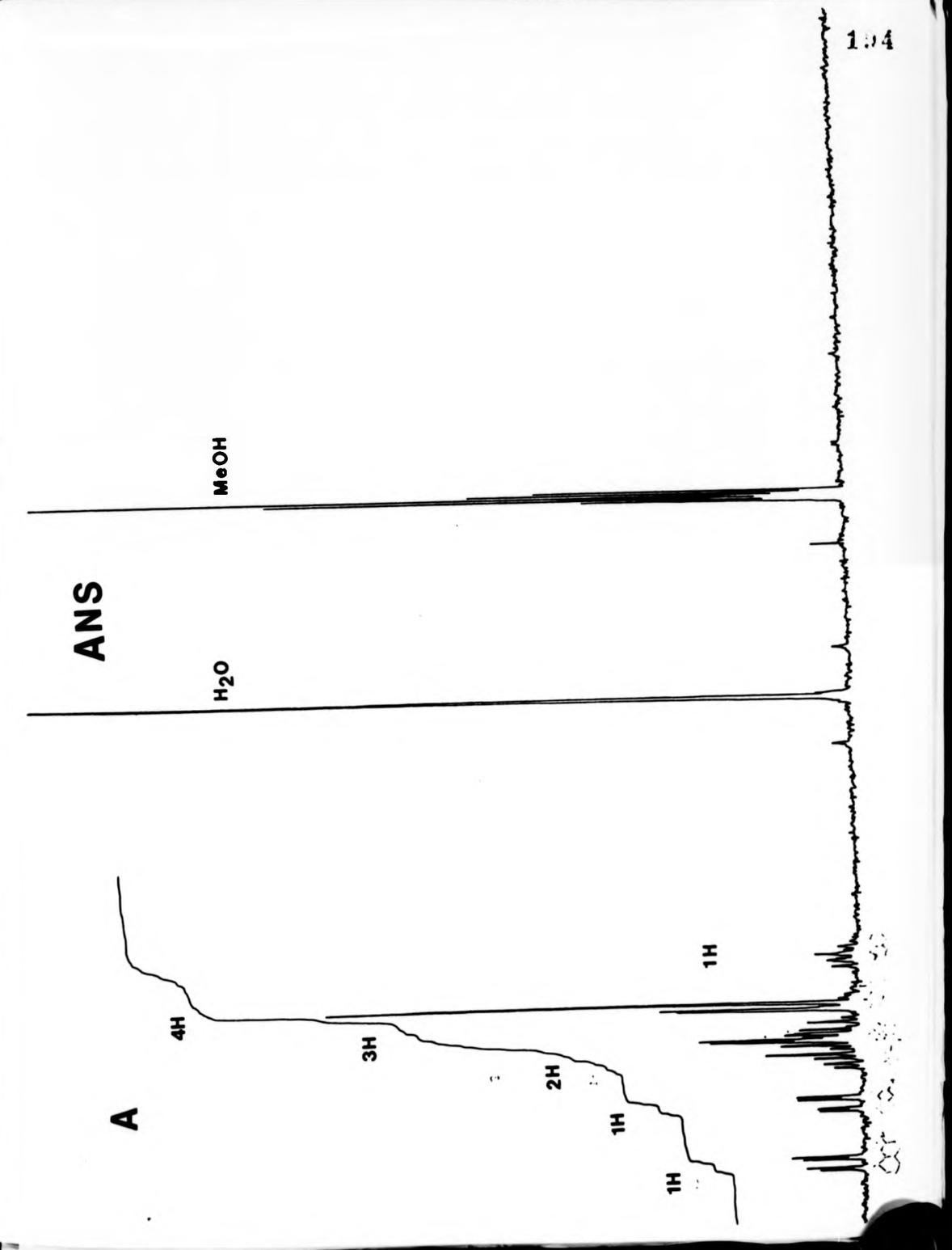
^{1}H 90MHz Spectra

Legend:

A. ^{1}H 90MHz spectrum of 1-analino-8-napthalene sulphonate (ANS), 10mg/ml.

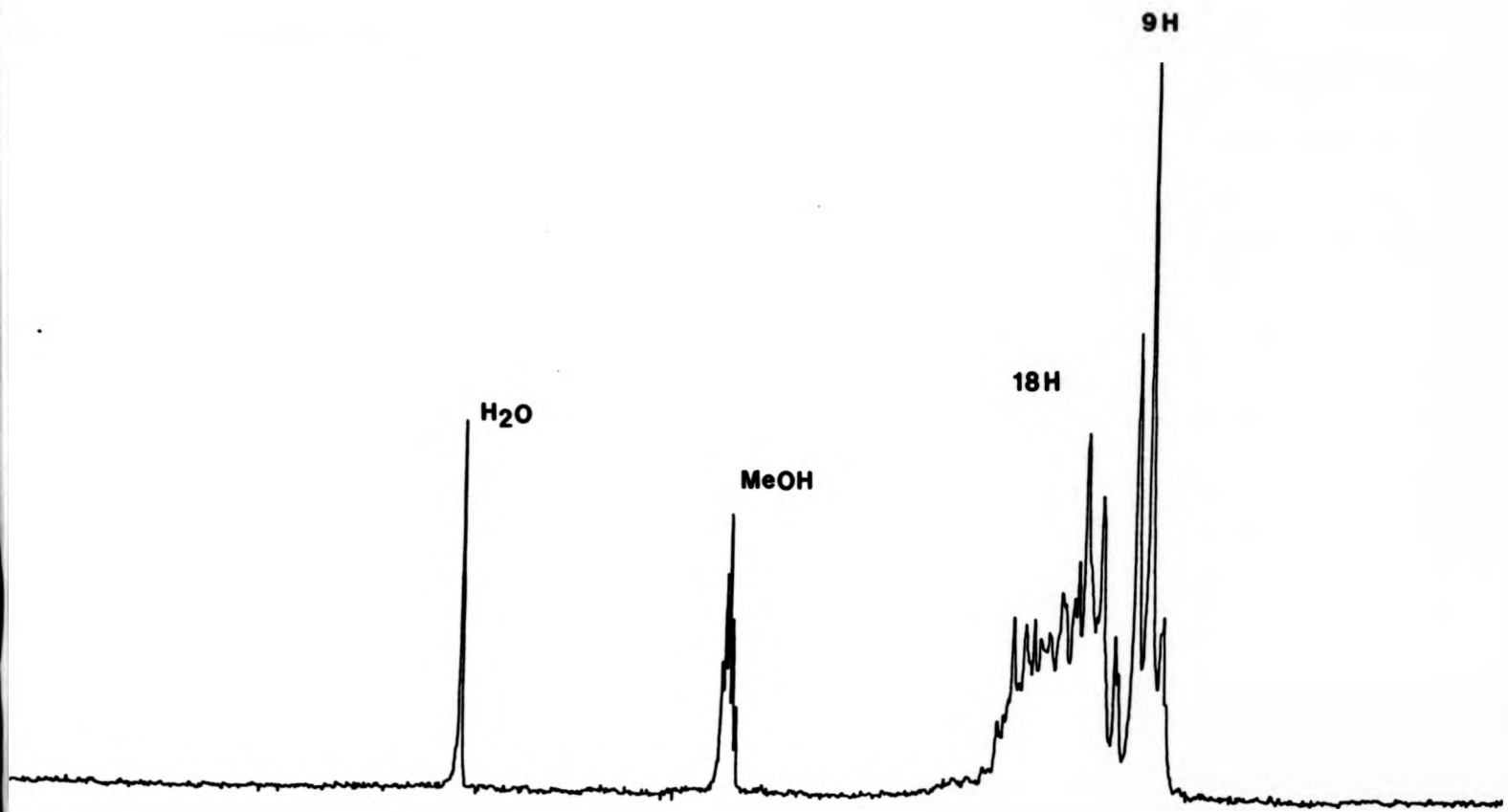
B. ^{1}H 90MHz spectrum of tributyltin chloride (TBT), 10mg/ml.

C. ^{1}H 90MHz spectrum of ANS + TBT complex, 10mg/ml.

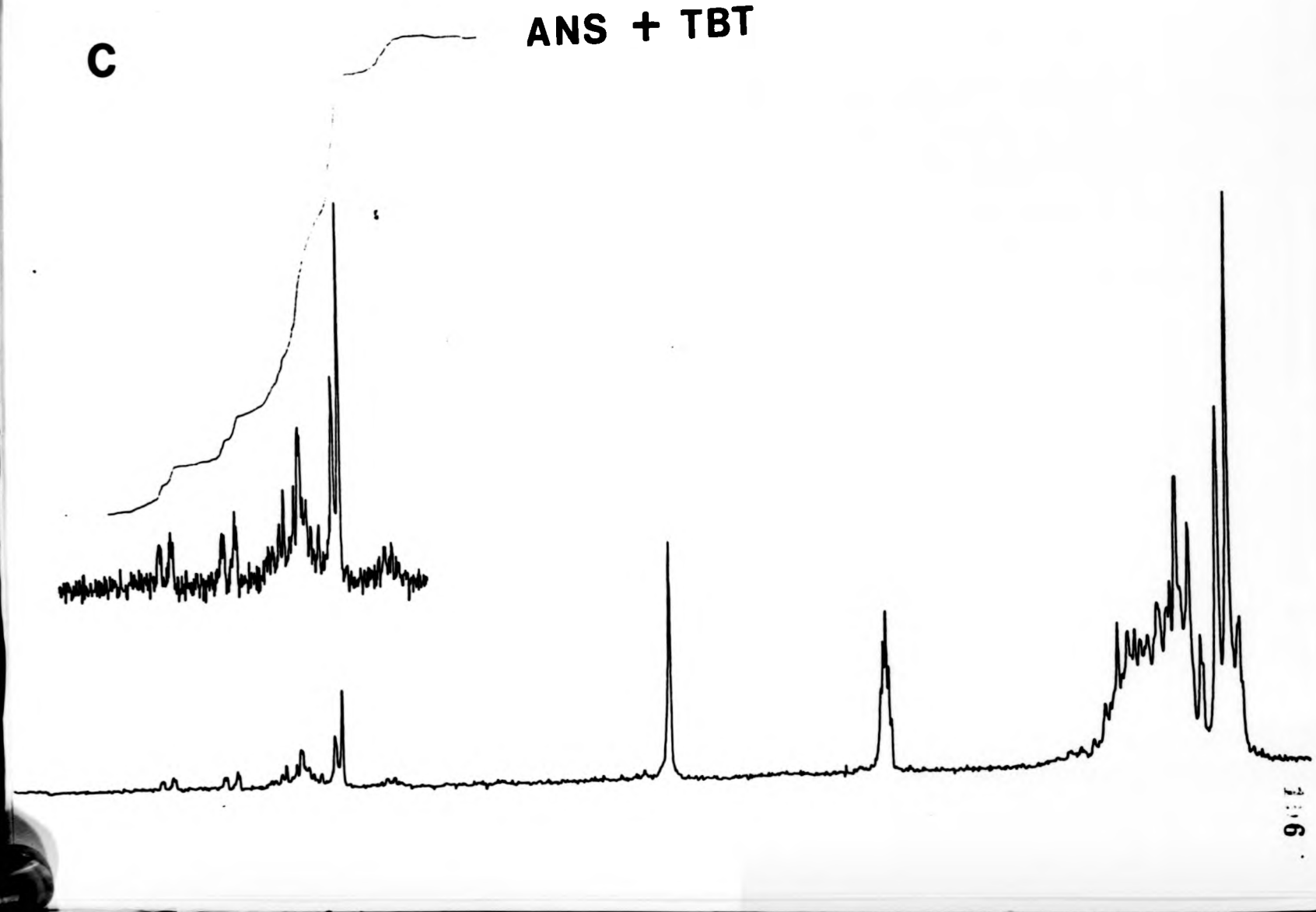


B

TBT



1.5


C
ANS + TBT

diluted as described in 'General Procedures'. Ethanol was never allowed to exceed 1% of the total solvent. Table 1Ai provides the linear concentration ranges and detection limits of the method under the above conditions for various triorganotins.

Table 1Ai demonstrates the applicability of the method to mono, di- and triethyltin chlorides, although a reduction in the sensitivity is experienced with the loss of consecutive n-alkyl groups. Most sensitive under these conditions is the detection of n-trialkyltins. Varying the anion of the tributyltin derivatives appears to make little difference to the linear concentration ranges with these compounds. This phenomenon provides for the advantage of allowing the investigator to assay alkyltins at various stages of degredation and/or metabolism, when applied to test samples. However, the detection of specific trialkyltin compounds can only be attempted with a priori knowledge of the anion under investigation.

It must also be noted that the assay does not extend to aryltins, since neither triphenyltin chloride, nor the penta-coordinate complex 2-((dimethylamino) methyl) phenyl diethyltin chloride, respond in this system.

Recovery from and the Determination of Trialkyltins, in the Presence of Biological Samples

The assay provides the flexibility of allowing the investigator to make quick estimations in aqueous suspensions of biological samples, or allowing extraction and concentration with n-hexane before determination.

Exogenous tributyltin chloride was added to rat liver homogenates and the samples diluted and assayed for alkyltin concentration in aqueous suspension, as described in the 'General Procedures'. The detection level of such a procedure was 10×10^{-6} M final concentration

Table 1Ai

Typical Conditions Employed to Measure μM Quantities of n-alkyltin

Compound	Wavelength(nm)		Conc. Range	Detection Limit
	Ex	Em	($1x10^{-6}$M)	($1x10^{-6}$M)
Me_3SnCl	380	460	2.0- 80	1.0
$EthSnCl_3$	380	460	5.0- 25	5.0
Eth_2SnCl_2	380	460	2.0- 20	2.0
Eth_3SnCl	380	460	2.0-100	1.0
Pr_3SnCl	375	455	1.0-100	0.2
Bu_3SnCl	374	455	0.5-100	0.1
$(Bu_3Sn)_2O$	374	455	1.0-100	0.5
$(Bu_3Sn)_2PO_4$	374	455	1.0-100	0.5
Bu_3Sn $CO_2C_2H_2CO_2CH_3$	374	455	1.0-100	0.5
Oct_3SnCl	370	460	3.0- 50	1.0
Cy_3SnCl	370	460	5.0- 50	1.0
Ph_3SnCl	380	460	-	-

or 30 nmoles of tributyltin chloride.

However, if the n-hexane extraction procedure of Arakawa et al, 1981b, was pursued some 92% ± 1 (the average and deviation of five estimates) of the total tributyltin chloride added was recoverable, allowing 3.5 ± 1.0 nmoles of the alkyltin to be detectable in total samples.

It is proposed that the above method should provide a useful, reliable and rapid assay for various n-alkyltins.

APPENDIX 2

Toxicological Studies with Organotins

Investigations of the Fungistatic and Bacteriostatic Activities of Organotins

As discussed earlier, the use of organotins as biocides has increased in recent years, and has diverged with more varied methods of synthesis of functionally substituted organotin compounds. The use of these compounds has been reviewed and commented upon by several authors: Stapher, 1969; Evans and Smith, 1975; and Smith, 1982. Presented here are a series of results based on ester tin derivatives synthesised at Akzo Chemie UK with a view to finding fresh alternatives with which to continue functional studies.

In previous studies the basic n-alkyltins have almost always proved to be the best formula, and the model compound n-tributyltin oxide has, similarly, proved intransient in its structure when attempting to modify its biocidal activities, (Kerk et al, 1954, 1956, 1957, 1959a, b, c, and d). This approach has indeed been disappointing. However, such studies have confirmed that certain organotins used as PVC stabilizers in contact with edible goods have low toxicity (Penninks and Seimen, 1982), and occasionally have identified useful compounds, for example, the penta-coordinate species Ve2283 (Aldridge, 1978).

The following results are based on minimum inhibitory concentration values (MIC-values) which prevent growth under stringent fermentative and oxidative conditions of obligate aerobes. The MIC-values are in parts per million (ppm) and were produced using a Dynatech MIC-2,000 system, which is located in Duren, West Germany. The test organisms employed were two strains of yeast (D22 and D27) of the species Saccharomyces cerevisiae and the Gram positive and negative bacteria

Bacillus subtilis and Escherichia coli respectively (ATC 1831, K12). The two modes of growth provide diagnostic evidence as to the mode of action of the inhibitor under test. Trialkyltins and uncouplers in general, are more potent under oxidative conditions, which is compatible with their in vitro activities on various biosystems energy conservation functions. The use of such drugs under these conditions has allowed the isolation resistant mutants directed at bioenergetic functions (Griffiths, 1976). The yeast culture media employed has been described earlier in this thesis, and Merck Casoboullion was used to culture both species of bacterium. Tables 2A 1, 2, 3, 4 and 5 represent MIC-values of various organotins grouped together under similar basic chemical structures.

As with other studies cited above, the functional substitution of alkyltins appears to reduce their biocidal activities. Such results taken as a whole, suggest that high biological activity is by no means a rule, but rather the exception, a situation which is in direct contrast to that of other heavy metals of which organic derivatives are documented eg. lead, mecury and antimony.

Despite the above general conclusion, it is noteworthy that this study has identified a compound (Table 2A3.4, di-n-butylchlorotin malate) which has a bacteriostatic activity, similar to that of n-tributyltin oxide (30ppm) in commercial use. It affects yeast at 30ppm which is half the concentration of any other compound studied here, but still six times that recorded for n-tributyltin oxide under similar conditions. Unlike n-tributyltin oxide, the compound has the same activity independent of the mode of growth, whereas most n-alkyltins show a 5 - 10 fold difference in active concentration between oxidative and fermentative growth conditions. The presence of the malate function in general, appears to affect the toxicity pattern as observed in

TABLE 1

Products

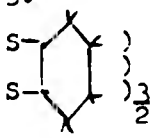
(BuAc) Sn R_3	D22 Ox	D22 Fm	D27 Ox	D27 Fm	E.coli Ox	E.coli Fm	B.subtilis Ox	B.subtilis Fm
1. $SCH_2CHOHCH_2OPh$	125	125	250	125-250	250	250-500	125-250	500
2. $CO_2CH{=}CHCO_2CH_3$	1000	250	1000	500	500	1000	500-1000	1000
3. 	125	250	250	500	1000	>1000	1000	>1000

TABLE 2

Products

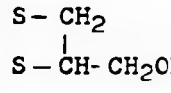
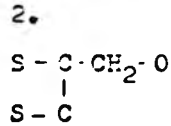
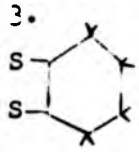
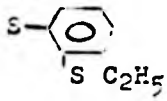
$(BuAc)_2$ Sn R_2	D22 Ox	D22 Fm	D27 Ox	D27 Fm	E.coli Ox	E.coli Fm	B.subtilis Cx	B.subtilis Fm
1. 	167	167	167	375	750	750	750	750
2. 	-	250	-	250	-	-	-	-
3. 	125	1000	250	1000	250	500	250	250
4. 	500	>500	>1000	>1000	>500	>500	250	500
5. =S	500	>500	500	>500	>500	>500	>500	>500

TABLE 2 (Cont'd)

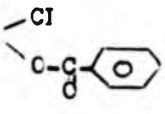
6. $SCH_2CO_2CH_3$	500	250	250	250	30-60	30-60	125-250	250
7. $SCH_2CH_2CO_2$	125	125	250	500	1000	500	1000	1000
8. $S\text{-}\underset{\underset{S}{\|}}{C}\text{-}O\ C_4H_9$	250	>1000	500	>1000	1000	500	1000	1000
9. $SCH_2CO_2CH_2\text{-}\underset{\underset{C_2H_5}{\|}}{\overset{\overset{H}{\|}}{C}}\text{-}C_4H_9$	250	500	250	500	250	500	250	500
10. $SCH_2\ CO_2CH_2CH_2OC_4H_9$	375	750	750	187	375	750	375	375
11. $SCH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}OCH{=}CHO\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_2SH_2$	500	>1000	1500	>1000	>1000	>1000	>1000	>1000
12. 	250	250	1000	500	500	500	250	250

TABLE 2 (cont'd)

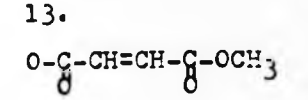
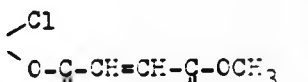
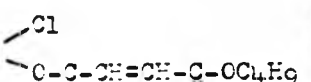
13. 	250	250	500	500	250	500	250	500
14. 	500	125	1000	250	500	500	250	250
15. 	250	250	1000	250	500	500	250	250

TABLE 3

Products $(BuAc)_3$ Sn R	D22 Ox	D22 Fm	D27 Ox	D27 Fm	E.coli Ox	E.coli Fm	B.subtilis Ox	B.subtilis Fm
1. PO_4^{2-}	187	93	187	650	750	375	375	375
2. O-C(=O)-CH_2-SH	187	375	187	375	375	750	375	750
3. CO_2-C(H)(CH_3)-O-(2,4-dichlorophenyl)	750	1500	1500	1500	750	1500	1500	1500
4. HO-CO_2-(phenyl)	1000	1000	1000	>1000	>1000	1000	1000	1000
5. CO_2CHOH-(phenyl)	250	125	250	500	500	250	250	500
6. CH_3-C(=O)-O-(phenyl)-CO_2-	750	375	1500	375	1500	1500	1500	1500

TABLE 4

Products	D22		D27		E.coli		B.subtilis	
Cl_2 Bu-Sn-R	Ox	Fm	Ox	Fm	Ox	Fm	Cx	Fm
1. $SCH_2CO_2CH_2-C(C_2H_5)_2-C_3H_7$	250	250	125	250	500	500	250	500
2. $SCH_2CO_2CH_2C_5H_{11}$	250	250	125	250	1000	500	1000	500
3. Cl-Sn(-S-CH_2)(-O-C(=O))-	250	125	500	250	250	500	250	500
4. O-C(=O)-C=C-C(=O)-O-CH_3	1000	250	1000	500	250	250	125	125
5. $(O-C(=O)-C=C-C(=O)-O-CH_3)_2$	500	250	500	500	250	250	125	125

TABLE 5

Products

$Bu_2^{Cl}Sn-R$	D22 Ox	D22 Fm	D27 Ox	D27 Fm	E.coli Ox	E.coli Fm	B.subtilis Ox	B.subtilis Fm
1. $S-(CH_2)_{11}-CH_3$	500	1000	1000	1000	1000	500	1000	500
2. $SCH_2CO_2CH_2\underset{\displaystyle C_2H_5}{\underset{\vert}{C}}HC_4H_9$	375	1500	750	1500	750	750	750	750
3. $O\overset{\displaystyle O}{\overset{\Vert}{C}}-(CH_2)_{10}-CH_3$	500	1000	500	1000	1000	500	1000	500
4. $O\overset{\displaystyle O}{\overset{\Vert}{C}}-CH=CH-\overset{\displaystyle O}{\overset{\Vert}{C}}-O-CH_3$	30-60	30	60	30-60	25-50	25-50	25-50	25-50
5. $O\overset{\displaystyle O}{\overset{\Vert}{C}}-CH=CH\overset{\displaystyle O}{\overset{\Vert}{C}}-O-Sn(Cl)-Bu_2$	60	60	250	250	60-125	30-60	30-60	30-60
6. $O\overset{\displaystyle O}{\overset{\Vert}{C}}-\underset{\displaystyle R_3}{\underset{\vert}{\overset{\displaystyle R_1}{\overset{\vert}{C}}}}-R_2$ (C_8-C_{12})	60	30	250	250	60-125	60-125	30-60	60-125

TABLE 5 (Cont'd)

7. $(O-\overset{\overset{O}{\|}}{C}-CH=CH-\overset{\overset{O}{\|}}{C}-O-C_8H_{17})_2$	90-187	187-375	375	375	90-187	187	90-187	187
8. $(O-\overset{\overset{O}{\|}}{C}-C\equiv C-\overset{\overset{O}{\|}}{C}-O-CH_3)_2$	60-125	125-250	500	500	125	250	250	500
9. $(O-\overset{\overset{O}{\|}}{C}-CH=CH-\overset{\overset{O}{\|}}{C}-O-C_4H_9)_2$	187	187	187	187	90-180	180	90-180	180

Tables 2A4 and 5. The structure activity relationship of the malate substitution may only be speculated upon at this time. As malate is a well characterised metabolite, it may provide a recognisable group for cell transport, although upon entry, its mode of action is certainly more diverse than n-alkyltins; as the cells' mode of growth (oxidative or fermentative) has no impact on the substituted compound's activity. Preliminary results suggest that similar to n-alkyltins, n-dibutylchlorotin malate will inhibit energy linked functions in vitro, but other effects are also expected.

Electron Micrographic Evidence for the Macro Action of Triorganotins

Aerobic cultures of the yeast Saccharomyces cerevisiae (D27) have been subjected to increasing concentrations of n-tributyltin oxide (both lethal and sublethal) and the cell surface of these specimens observed by scanning electron microscopy.

The experimental cells were grown to exponential phase under oxidative conditions, before treatment with n-tributyltin oxide, then incubated for a further 4 hours before the samples were diluted and plated to assess cell viability. The remaining cells were pelleted in a bench centrifuge (2 k x g) and resuspended in 25% glutaldehyde for 30 seconds fixation. The cells were then washed 3 times in sterile distilled water before the samples were mixed with acetone and fixed to sample stages. The specimens were then gold shadowed for 30 seconds, before inspection under the scanning electron microscope.

Presented overleaf are a series of electronmicrographs produced using the above protocol.

Figure 2A.i

A. Control Cells in the Absence of Tributyltin Oxide

Legend:
The control cells opposite show healthy yeast cells demonstrating budding (centre right) and the residual bud scars of the parent (centre). One cell in view shows a crumpled effect which is presumed to be a deformation due to fixation.

B. Cells Treated with 3ppm of Tributyltin Oxide

Legend:
The cells opposite display swelling and a certain degree of cell breakage. Cell leakage is visible in the centre of the electron-micrograph. Samples of these cells taken prior to fixation showed 55% viability compared with the control samples.

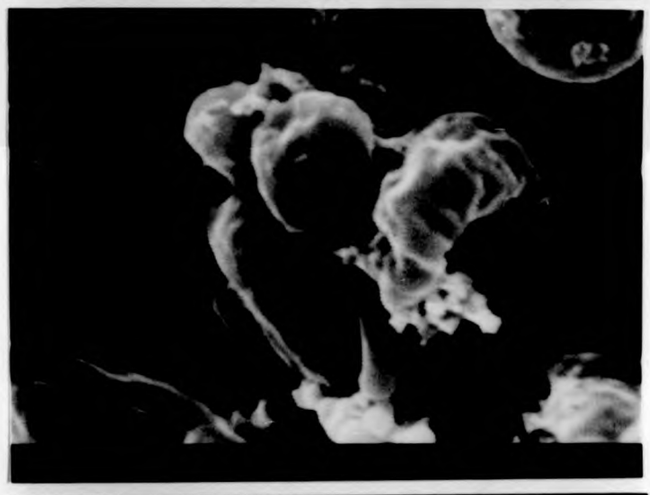
C. Cells Treated with 5ppm of Tributyltin Oxide

Legend:
The cell pictured in Figure 2A.iC has completely ruptured due to swelling. It has swollen to some 4 or 5 times the size of the healthy cells and the cell surface also appears to be severely disrupted. Cells subjected to these conditions are only some 5% viable and further lethality is envisaged with prolonged exposure.

In general, cells exposed to trialkyltin appear to undergo gross swelling before cell breakage and ultimately, cell death.

A

B



C

Figure 2A.i

A. Control Cells in the Absence of Tributyltin Oxide

Legend:
The control cells opposite show healthy yeast cells demonstrating budding (centre right) and the residual bud scars of the parent (centre). One cell in view shows a crumpled effect which is presumed to be a deformation due to fixation.

B. Cells Treated with 3ppm of Tributyltin Oxide

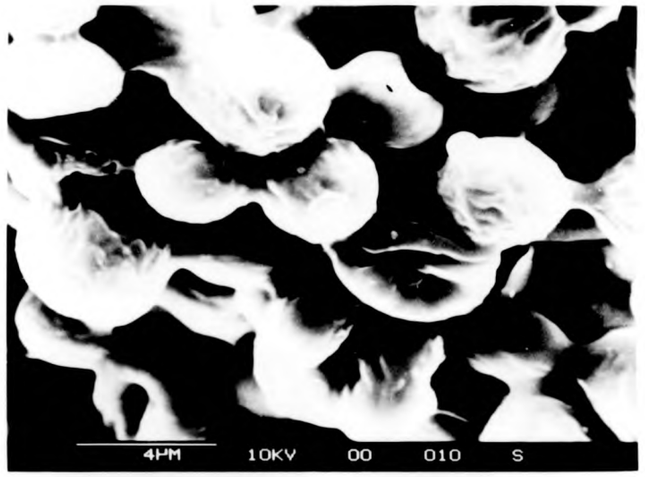
Legend:
The cells opposite display swelling and a certain degree of cell breakage. Cell leakage is visible in the centre of the electron-micrograph. Samples of these cells taken prior to fixation showed 65 viability compared with the control samples.

C. Cells Treated with 5ppm of Tributyltin Oxide

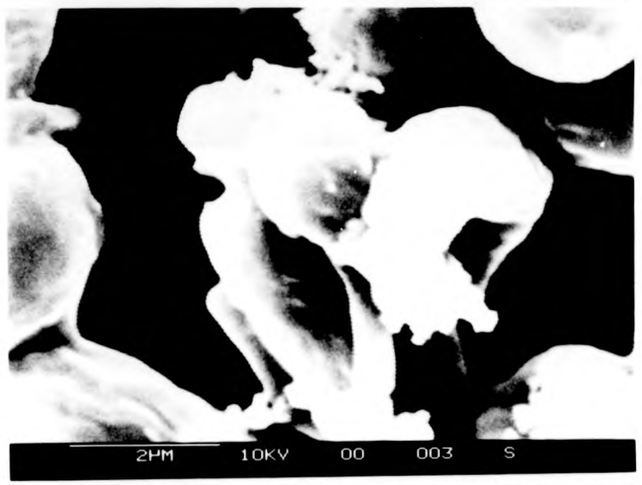
Legend:
The cell pictured in Figure 2A.iC has completely ruptured due to swelling. It has swollen to some 4 or 5 times the size of the healthy cells and the cell surface also appears to be severely disrupted. Cells subjected to these conditions are only some 5 viable and further lethality is envisaged with prolonged exposure.

In general, cells exposed to trialkyltin appear to undergo gross swelling before cell breakage and ultimately, cell death.

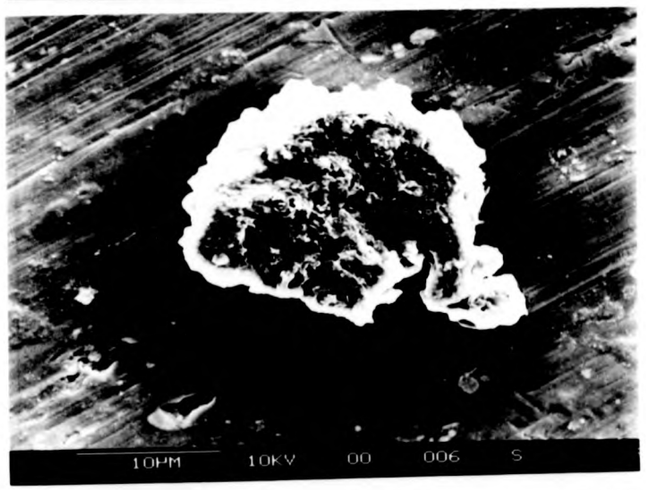
A



B



C



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